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**TITLE**

INTERFERON MODULATION OF T-CELL RESPONSES TO  
SEMLIKI FOREST VIRUS INFECTED MURINE BRAIN CELLS

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**INSTITUTION  
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1989

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INTERFERON MODULATION OF T-CELL RESPONSES TO  
SEMLIKI FOREST VIRUS INFECTED MURINE BRAIN CELLS

by

Paul Thomas Tomkins BSc Hons (Warwick)

A thesis submitted for the degree of

Doctor of Philosophy

of the

University of Warwick

Department of Biological Sciences

University of Warwick

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## TABLE OF CONTENTS

Contents	i
Acknowledgements	vii
Declaration	viii
Dedication	ix
List of photographs	x
List of figures	x
List of tables	xv
Abbreviations	xvii
List of publications	xx
Summary	xxi

### Chapter 1: Introduction

Prelude	1
(1) Introduction to the interferon system	1
(2) Mechanisms of the antiviral effect of interferon on cells	7
(3) Importance of major histocompatibility complex antigens in the immune system.	11
(a) Introduction to the major histocompatibility complex antigens	11
(b) Interferon modulation of MHC antigen expression	14
(c) Importance of class I MHC antigens for cytotoxic T-lymphocyte recognition of target cells	22
(d) Importance of class II MHC antigens for helper-T-lymphocyte function.	30
(4) Mechanisms of cytotoxic T-lymphocyte killing of target cells	36
(5) Role of interferon <i>in vivo</i>	42

(6) Semliki Forest virus	
(a) History, classification, structure and replication	46
(b) SFV-infection of the laboratory mouse	48
(7) Aims of project and introduction to the experimental system	54
 <u>Chapter 2: Materials and Methods</u>	 58
 <u>Materials</u>	 58
(A) Medium	58
(B) Cell lines	58
(C) Virus	61
(D) Mice	61
(E) Interferons	62
(F) Antibodies	62
(G) Radiochemicals	65
(H) Other materials	65
(I) Buffers and solutions	67
(J) Name and address of suppliers	68
 <u>Methods</u>	 71
(A) Production and assay of SFV	71
(B) Preparation of $\beta$ -propiolactone inactivated SFV (BPLSFV)	72
(C) Inoculation of mice with virus:	73
(i) Intranasal	73
(ii) Intraperitoneal	73
(D) Administration of interferon gamma and antibodies to interferon gamma to mice	74

(E) Harvesting of mouse tissues:	74
(i) Serum	74
(ii) Brain	75
(F) Preparation of primary brain cell cultures from newborn C3H/He mice	75
(G) Procedure for indirect immunofluorescence staining of intracellular glial fibrillary acidic protein and fibronectin for observation by UV microscopy	76
(H) Indirect immunofluorescence staining of cell surface antigens and quantification by flow cytometry:	77
(i) Interferon treatment of cells and staining of cell surface antigens by indirect immunofluorescence	78
(ii) Analysis of data	80
(iii) Titration of antibody preparations	81
(I) Production of monoclonal antibody supernatant and ascites fluid	81
(J) Preparation of SFV-specific effector T-cells	83
(K) Preparation of alloreactive effector T-cells	84
(L) Harvesting of lymphocytes for assay of non-specific cytotoxicity	85
(M) Cytotoxicity assays	85
(N) Assay to study the ability of SFV-treated astrocytes to stimulate SFV-specific T-cells to proliferate and release IFN- $\gamma$	88
(O) Alloproliferation assays	90
(P) Interferon assay	90
(Q) Determination of relative sensitivity of brain cells to interferon	94
(R) Storage of cells in liquid nitrogen	95

<u>Chapter 1: Replication of SFV in brain cells</u>	96
Introduction	96
Results:	97
(A) Production and partial characterisation of primary brain cell cultures prepared from newborn C3H/He mice	97
(B) Replication of SFV in astrocytes, G26-24 and C1300 cells	98
Discussion	101
<u>Chapter 4: Effect of IFN on SFV replication and MHC antigen display by brain cells</u>	103
Introduction	103
Results:	104
(A) Relative sensitivity of brain cells to IFN- $\alpha\beta$ and IFN- $\gamma$	104
(B) Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on SFV antigen expression by SFV-infected cells	105
(C) Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on expression of class I and class II MHC antigen by brain cells	111
(D) Interaction of IFNs $\alpha$ , $\beta$ and $\gamma$ in the induction of MHC antigen on astrocytes	123
Discussion	126
<u>Chapter 5: Effect of IFN on the susceptibility of brain cells to SFV-specific cytotoxic T-lymphocyte lysis</u>	138
Introduction	138



## Results:

139

- (A) Effect of IFN treatment on susceptibility of astrocytes to SFV-specific cytotoxic T-lymphocyte lysis 139
- (B) Effect of SFV-infection and BPLSFV-treatment on class I and class II MHC antigen expression by astrocytes 141
- (C) Effect of IFN treatment on susceptibility of G26-24 cells to SFV-specific cytotoxic T-lymphocyte lysis 144
- (D) Effect of IFN treatment on susceptibility of C1300 cells to SFV-specific cytotoxic T-lymphocyte lysis 146

## Discussion:

150

- (A) Effect of IFN treatment on the susceptibility of SFV-infected brain cells to SFV-specific CTL lysis 150
- (B) Genetic mapping of the SFV-specific CTL response 160

## Chapter 4: Effect of IFN on the ability of brain cells to present SFV antigen to SFV specific T-cells as assessed by proliferation and the release of IFN- $\gamma$

164

## Introduction

164

## Results:

165

- (A) Effect of IFN on the ability of astrocytes to present SFV antigen to SFV-specific T-cells 165
- (B) Effect of IFN on the ability of BPLSFV-treated astrocytes and BPLSFV-treated G26-24 cells to stimulate SFV-specific T-cell release of IFN- $\gamma$  166

## Discussion

177

<u>Chapter 7: Presence of IFN within the brains of SFV-infected C3H/He mice, effect on brain cell MHC antigen expression and preliminary studies using BA-6A2 monoclonal antibody to determine the role of endogenously produced IFN-<math>\gamma</math></u>	185
Introduction	185
Results:	187
(A) Presence of IFN within the brains of SFV-infected C3H/He mice and effect on brain cell MHC antigen expression	187
(B) Preliminary studies using the BA-6A2 rat-anti-murine IFN- $\gamma$ monoclonal antibody to determine the role of IFN- $\gamma$ produced endogenously during SFV-infection of mice	192
Discussion:	196
(A) Presence of IFN within the brains of SFV-infected C3H/He mice and effect on brain cell MHC antigen expression	196
(B) Preliminary studies using BA-6A2 anti-IFN- $\gamma$ monoclonal antibody to determine the role of IFN- $\gamma$ produced endogenously during SFV-infection of C3H/He mice	201
<u>Chapter 8: Final discussion</u>	206
<u>References</u>	219

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#### Declaration

All of the results presented in this thesis were obtained entirely by myself with the exception of those presented in figure 4.7 in chapter 4 which were obtained by Mr M J Blackman (National Institute for Medical Research, London). Photographs 3.1 and 3.2 in chapter 3 were taken by Mr R Ling (University of Warwick).

To my family

#### List of photographs

3.1	Primary brain cell culture prepared from the brains of newborn C3H/He mice, phase contrast	97
3.2	Primary brain cell culture stained with antiserum to glial fibrillary acidic protein by indirect immunofluorescence	97

#### List of figures

1.1a	Structure of the murine class I MHC H-2K <sup>b</sup> antigen	12
1.1b	Three dimensional structure of human class I MHC HLA-A2 antigen	12
1.2	Structure of class II MHC antigens	13
3.1	SFV antigen expression by infected astrocytes	99
3.2	SFV antigen expression by infected G26-24 and C1300 cells	99
4.1	Effect of IFN- $\alpha\beta$ on incorporation of [ <sup>3</sup> H]uridine into SFV-RNA by astrocytes and L929 cells	104
4.2	Effect of IFN- $\gamma$ on incorporation of [ <sup>3</sup> H]uridine into SFV-RNA by astrocytes and L929 cells	104
4.3	Effect of IFN- $\alpha\beta$ on incorporation of [ <sup>3</sup> H]uridine into SFV-RNA by G26-24 cells and L929 cells	104
4.4	Effect of IFN- $\gamma$ on incorporation of [ <sup>3</sup> H]uridine into SFV-RNA by G26-24 cells and L929 cells	104
4.5	Effect of IFN- $\alpha\beta$ on incorporation of [ <sup>3</sup> H]uridine into SFV-RNA by C1300 cells and L929 cells	104
4.6	Effect of IFN- $\gamma$ on incorporation of [ <sup>3</sup> H]uridine into SFV-RNA by C1300 cells and L929 cells	105
4.7	Effect of IFN- $\alpha\beta$ on expression of SFV antigen by SFV-infected astrocytes	106

4.8	Effect of IFN- $\gamma$ on expression of SFV antigen by SFV-infected astrocytes	106
4.9	Effect of IFN- $\gamma$ on expression of SFV antigen by BPLSFV-treated and SFV-infected G26-24 cells	108
4.10	Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ on expression of SFV antigen by BPLSFV-treated and SFV-infected C1300 cells	109
4.11	Effect of IFN- $\alpha\beta$ on expression of class I MHC H-2D <sup>k</sup> antigen by astrocytes	112
4.12	Effect of IFN- $\gamma$ on expression of class I MHC H-2D <sup>k</sup> antigen by astrocytes	112
4.13	Effect of IFN- $\alpha\beta$ on expression of class I MHC H-2K <sup>k</sup> antigen by astrocytes	113
4.14	Effect of IFN- $\gamma$ on expression of class I MHC H-2K <sup>k</sup> antigen by astrocytes	113
4.15	Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on susceptibility of astrocytes to lysis by alloreactive CTL	114
4.16	Effect of IFN- $\gamma$ on expression of class II MHC H-2A <sup>k</sup> antigen by astrocytes	114
4.17	Effect of IFN- $\alpha\beta$ on expression of class II MHC H-2A <sup>k</sup> antigen by astrocytes	114
4.18	Effect of IFN- $\alpha\beta$ on expression of class II MHC H-2D <sup>b</sup> antigen by G26-24 cells	115
4.19	Effect of IFN- $\gamma$ on expression of class I H-2D <sup>b</sup> antigen by G26-24 cells	115
4.20	Effect of IFN- $\alpha\beta$ on expression of class I MHC H-2K <sup>b</sup> antigen by G26-24 cells	116
4.21	Effect of IFN- $\gamma$ on expression of class I MHC H-2K <sup>b</sup> antigen by G26-24 cells	116

4.22	Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on susceptibility of G26-24 cells to lysis by alloreactive CTL	117
4.23	Effect of IFN- $\gamma$ on expression of class II MHC H-2A <sup>b</sup> antigen by G26-24 cells	118
4.24	Effect of IFN- $\alpha\beta$ on expression of class II H-2A <sup>b</sup> antigen by G26-24 cells	118
4.25	Effect of IFN- $\alpha\beta$ on expression of class I MHC H-2D <sup>d</sup> antigen by C1300 cells	119
4.26	Effect of IFN- $\gamma$ on expression of class I MHC H-2D <sup>d</sup> antigen by C1300 cells	119
4.27	Effect of IFN- $\alpha\beta$ on expression of class I MHC H-2K <sup>k</sup> antigen by C1300 cells	120
4.28	Effect of IFN- $\gamma$ on expression of class I MHC H-2K <sup>k</sup> antigen by C1300 cells	120
4.29	Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on susceptibility of C1300 cells to lysis by alloreactive CTL	121
4.30	Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ on expression of class II MHC H-2A <sup>k</sup> antigen by C1300 cells	122
4.31	Effect of IFN- $\alpha\beta$ on the augmentation by IFN- $\gamma$ of class II MHC H-2A <sup>k</sup> antigen on astrocytes	124
4.32	Effect of 2-day pretreatment with IFN- $\alpha\beta$ on the augmentation by IFN- $\gamma$ of class II MHC H-2A <sup>k</sup> antigen on astrocytes	124
4.33	Effect of IFN- $\beta$ on the augmentation by IFN- $\gamma$ of class II MHC H-2A <sup>k</sup> antigen on astrocytes	125
5.1	Effect of IFN- $\alpha\beta$ on susceptibility of SFV-infected astrocytes to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement	139



5.2	Effect of IFN- $\gamma$ on susceptibility of SFV-infected and BPLSFV-treated astrocytes to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement	140
5.3	Effect of 4-day treatment of astrocytes with BPLSFV on class II MHC H-2A <sup>k</sup> antigen expression	143
5.4	Spontaneous release of <sup>51</sup> chromium from SFV-infected G26-24 cells and lysis by anti-SFV serum in the presence of complement	145
5.5	Effect of IFN- $\gamma$ on susceptibility of SFV-infected and BPLSFV-treated G26-24 cells to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement	145
5.6	Effect of IFN- $\gamma$ on susceptibility of SFV-infected and BPLSFV-treated C1300 cells to lysis by C3H/He (H-2 <sup>k</sup> ) SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement	147
5.7	Effect of IFN- $\gamma$ on susceptibility of SFV-infected C1300 cells to lysis by A/J (H-2 <sup>d</sup> ) SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement	148
6.1	Production of IFN- $\alpha\beta$ by SFV-infected and BPLSFV-treated astrocyte cultures	169
6.2	Effect of anti-IFN- $\gamma$ antibody on ability of supernatant harvested from co-cultures of IFN- $\gamma$ treated BPLSFV-treated astrocytes with SFV-T-cells to augment class II H-2A <sup>k</sup> antigen expression on astrocytes	170
6.3	Effect of anti-IFN- $\gamma$ antibody on ability of supernatant harvested from co-cultures of IFN- $\gamma$ treated BPLSFV-treated G26-24 cells and SFV-T-cells to augment class II MHC (H-2A <sup>b</sup> ) antigen expression on G26-24 cells	174

7.1	Effect of A7(74) SFV infection of newborn mice on class II and class II MHC antigen expression by cells within the brain	190
7.2	Effect of brain extracts from A7(74) SFV-infected newborn mice on class I and class II MHC antigen expression by cultured astrocytes	191
7.3	Effect of brain extracts from SFV-infected newborn and adult C3H/He mice on susceptibility of cultured astrocytes to alloreactive CTL lysis	192

# List of tables

3.1	RNA synthesis by SFV in astrocytes and L929 cells	98
3.2a	RNA synthesis by SFV in G26-24 and L929 cells	99
3.2b	RNA synthesis by SFV in C1300 and L929 cells	99
4.1	Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on ability of astrocytes to stimulate an allogeneic mixed lymphocyte reaction	115
4.2	Effect of IFN- $\alpha\beta$ on the augmentation by IFN- $\gamma$ of class I MHC H-2K <sup>k</sup> antigen on astrocytes	123
4.3	Effect of IFN- $\beta$ on the augmentation by IFN- $\gamma$ of class I MHC H-2K <sup>k</sup> antigen on astrocytes	123
5.1	Effect of BPLSFV-treatment and SFV-infection on class I MHC H-2D <sup>k</sup> antigen expression by astrocytes	142
5.2	Effect of BPLSFV-treatment and SFV-infection on class II MHC H-2A <sup>k</sup> antigen expression by astrocytes	143
6.1	Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on ability of BPLSFV-treated astrocytes to stimulate SFV-specific T-cell release of IFN- $\gamma$	166
6.2	Effect of supernatants harvested from co-cultures of astrocytes and SFV-specific T-cells on class II H-2A <sup>k</sup> antigen expression by astrocytes	169
6.3	Effect of supernatants harvested from co-cultures of astrocytes and SFV-specific T-cells on class I H-2K <sup>k</sup> antigen expression by astrocytes	171
6.4	Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on ability of BPLSFV-treated G26-24 cells to stimulate SFV-specific T-cell release of IFN- $\gamma$	173

6.5	Effect of supernatants harvested from co-cultures of G26-24 cells and SFV-specific T-cells on class II H-2A <sup>b</sup> antigen expression on G26-24 cells	174
6.6	Effect of supernatants harvested from co-cultures of G26-24 cells and SFV-specific T-cells on class I MHC H-2D <sup>b</sup> expression on G26-24 cells	175
7.1	Titres of IFN present in SFV-infected adult C3H/He mouse brain	187
7.2	Effect of brain extracts from A7(74) SFV-infected adult C3H/He mice on class I MHC H-2K <sup>k</sup> antigen expression by cultured astrocytes	188
7.3	Time course of titres of IFN- $\alpha\beta$ and SFV present in SFV-infected newborn C3H/He mouse brain	189
7.4	Persistence of R4-6A2 anti-IFN- $\gamma$ monoclonal antibody in the serum of adult C3H/He mice	192
7.5	Effect of anti-IFN- $\gamma$ antibody on number of C3H/He mice surviving infection with A7(74) SFV	193
7.6	Effect of IFN- $\gamma$ on the natural killer cell activity of C3H/He mouse splenic lymphocytes	194
7.7	Effect of anti-IFN- $\gamma$ antibody on ability of IFN- $\gamma$ to increase the natural killer cell activity of C3H/He mouse splenic lymphocytes	195

### Abbreviations

2-5A	: 2'-5'-oligoadenylate
$\text{\AA}$	: Angstrom ( $10^{-10}$ metres)
Ad	: Adenovirus
AED	: N-iodoacetyl-N-(5-sulfonic-1-naphthyl)-ethylene-diamine
AMD	: Actinomycin D
APBCs	: Antigen presenting B-lymphoma cells
ATCC	: American tissue culture collection
BHK	: Baby hamster kidney
$\beta_2M$	: $\beta_2$ -microglobulin
BPL	: $\beta$ -propiolactone
BPLSFV	: $\beta$ -propiolactone inactivated preparation of Semliki Forest virus
cDNA	: Copy deoxyribonucleic acid
Ci	: Curie
CPE	: Cytopathic effect
CPM	: Counts per minute
CTL	: Cytotoxic T-lymphocyte
DI	: Defective interfering
DMEM	: Dulbecco's modification of Eagles minimum essential medium
DTH	: Delayed type of hypersensitivity
EAE	: Experimental allergic encephalomyelitis
EDTA	: Diaminoethanetetra-acetic acid
Fc	: Fragment crystallizable (of immunoglobulin)
FCS	: Foetal calf serum
FITC	: Fluorescein isothiocyanate
FL	: Fluorescein
FL1	: Fluorescence intensity channel-1

GFAP	: Glial fibrillary acidic protein
GMEM	: Glasgow modification of Eagles minimum essential medium
H-2	: Murine major histocompatibility complex
HA	: Haemagglutinin
HCl	: Hydrochloric acid
HLA	: Human leukocyte antigen
Hrs	: Hours
ICAM-1	: Intercellular adhesion molecule-1
IFN	: Interferon
Ig	: Immunoglobulin
IL	: Interleukin
IRF	: Interferon regulatory factor
Kd	: Kilodalton
KLH	: Keyhole limpet hemocyanin
LCMV	: Lymphocytic choriomeningitis virus
LD <sub>50</sub>	: Lethal dose 50
LFA	: Lymphocyte function associated
MBP	: Myelin basic protein
MCMV	: Murine cytomegalovirus
MHC	: Major histocompatibility complex
MIR	: Mixed lymphocyte reaction
MLV	: Murine leukaemia virus
MSV	: Murine sarcoma virus
NaOH	: Sodium hydroxide
NBS	: Newborn calf serum
ND	: Not done
NEAA	: Non-essential amino-acids
NK	: Natural killer
nm	: Nanometres ( $10^{-9}$ metres)

NP	: Nucleoprotein
OVA	: Ovalbumin
PBL	: Peripheral blood lymphocytes
PBS	: Phosphate buffered saline
PFU	: Plaque forming unit
PGE	: Prostaglandin E
PPD	: Tuberculin-purified protein derivative
RNA	: Ribonucleic acid
dsRNA	: Double-stranded ribonucleic acid
RPM	: Revolutions per minute
RPNI	: Roswell Park Memorial Institute
SD	: Standard deviation
SFV	: Semliki Forest virus
SKSD	: Streptokinase streptodornase
SP	: 3-(p-sulphophenyldiazo)-4 hydroxy-phenylacetic acid
TNF	: Tumour necrosis factor
TNP	: Trinitrophenol
u/ml	: Units per millilitre
UV	: Ultra violet
WNV	: West Nile virus

List of publications

- (i) Morris, A.G., Tomkins, P.T., Maudsley, D.J. and Blackman, M.J. (1987). Infection of cultured murine brain cells by Semliki Forest virus : effects of interferon alpha/beta on viral replication, viral antigen display, major histocompatibility complex antigen display and lysis by cytotoxic T-lymphocytes. *Journal of General Virology* **68**, 99-106.
- (ii) Tomkins, P.T., Ward, G.A. and Morris, A.G. (1988). Role of interferon gamma in T-cell responses to Semliki Forest virus infected murine brain cells. *Immunology* **61**, 355-363.
- (iii) Maudsley, D.J., Morris, A.G. and Tomkins, P.T. (1989). Regulation by interferon of the immune responses to viruses via the major histocompatibility complex antigens. In : *Immune responses, virus infection and disease*. Edited by N.J. Dimmock and F.D. Minor. IRL Press, Oxford, UK. Society for General Microbiology Special Publication (In press).
- (iv) Morris, A.G. and Tomkins, P.T. (1989). Interactions of interferons in the induction of histocompatibility antigens in mouse fibroblasts and glial cells. *Immunology* (In press).



#### Summary

Cultures of astrocytes prepared from the brains of newborn mice, G26-24 oligodendrogloma cells and C1300 neuroblastoma cells were treated with interferon (IFN) and the effect on major histocompatibility complex (MHC) antigen expression assessed by indirect immunofluorescence. IFN- $\alpha\beta$  increased class I, but not class II, MHC antigen expression on astrocytes, G26-24 cells and C1300 cells. IFN- $\beta_1$  increased class I, but not class II, MHC antigen expression on astrocytes. IFN- $\gamma$  increased both class I and class II MHC antigen expression on astrocytes and G26-24 cells. IFN- $\gamma$  increased class I, but not class II, MHC antigen expression on C1300 cells. IFN- $\alpha\beta$  and IFN- $\beta_1$  were additive with IFN- $\gamma$  in the induction of class I MHC antigen expression on astrocytes, but inhibited the ability of IFN- $\gamma$  to induce class II MHC antigen expression. IFN- $\alpha\beta$  and IFN- $\gamma$  increased the susceptibility of astrocytes, G26-24 cells and C1300 cells to lysis by alloreactive cytotoxic T-lymphocytes (CTL) indicating that IFNs increased the ability of the cells to participate in class I MHC restricted T-cell immune reactions. Astrocytes treated with IFN- $\alpha\beta$  or IFN- $\gamma$ , and G26-24 cells and C1300 cells treated with IFN- $\gamma$ , prior to infection with Semliki Forest virus (SFV), showed a similar or increased susceptibility to SFV-specific CTL lysis, despite a reduction of SFV antigen display on the cells, as assessed by indirect immunofluorescence and susceptibility to lysis by anti-SFV antibody plus complement. It is concluded that even when SFV antigen expression is reduced by IFN treatment, in the context of enhanced class I MHC antigen expression cells remain susceptible to SFV-specific CTL lysis. IFN- $\alpha\beta$  and IFN- $\gamma$  treatment of astrocytes, and IFN- $\gamma$  treatment of G26-24 cells, prior to treatment with a  $\beta$ -propiolactone inactivated preparation of SFV, increased the ability of the cells to stimulate SFV-specific T-cell release of IFN- $\gamma$ . This increased ability correlated with an increase in MHC antigen expression on the cells. IFN- $\gamma$  released by SFV-specific T-cells increased class I and class II MHC antigen expression on astrocytes and G26-24 cells indicating that a positive feedback mechanism could operate. SFV-infected newborn and adult mice possessed high levels of IFN- $\alpha\beta$  in the brain. Brain extracts prepared from SFV-infected newborn and adult mice increased class I, but not class II, MHC antigen expression on astrocytes *in vitro*. Class I and class II MHC antigen expression was slightly elevated in the brains of SFV-infected newborn mice. To study the role of endogenous IFN- $\gamma$ , R4-6A2 anti-IFN- $\gamma$  monoclonal antibody was administered to adult mice, prior to infection with SFV, and the effect on the clinical course of SFV-disease monitored. R4-6A2 antibody had no effect and preliminary experiments indicated that the antibody may not neutralise all IFN- $\gamma$  activity *in vivo* under the conditions used.

## Chapter 1

### Introduction

Interferon (IFN) was discovered in 1957 by Isaacs and Lindenmann who observed that fluids harvested from cultures of chick chorio-allantoic membranes which had been treated with a heat-inactivated preparation of influenza virus contained a factor which could be recognised by its ability to render fresh cultures of the membranes resistant to infection by live influenza virus (Isaacs and Lindenmann, 1957). Since this time IFNs have been the subject of an immense number of studies, and it is now apparent that they have a wide range of activities including immunomodulatory effects which may, under certain circumstances, be of more importance than the classical antiviral effect.

The purpose of this introduction is not to give an exhaustive review of the IFN system, but rather to discuss those areas of IFN biology most relevant to this project and the possible physiological role of IFNs in viral disease. Since this project made use of the inbred mouse as a convenient model system on which the effects of IFN could be studied, much of what follows will be largely concerned with the murine system. However, since the long term hope of these studies is to facilitate the understanding of the role of human interferons in resistance to viral disease, comparisons with the human system will be made whenever differences between the systems are known to exist or as appropriate.

#### (1) Introduction to the interferon system

IFNs are defined as proteins which exert a virus non-specific antiviral

activity at least in homologous cells through cellular metabolic processes involving the synthesis of both ribonucleic acid (RNA) and protein (Committee on interferon nomenclature, 1980). To date three main classes of murine and human IFN have been identified;  $\alpha$ ,  $\beta$  and  $\gamma$ , which were classified on the basis of antigenicity using polyclonal antisera (Committee on interferon nomenclature, 1980). IFNs  $\alpha$  and  $\beta$ , also known as type I or viral IFNs, are produced by most cell types when infected with virus (De Maeyer, 1984). In contrast IFN- $\gamma$ , also known as type II or immune IFN, is produced exclusively by T-lymphocytes following stimulation by specific antigen or mitogen (Klein *et al.*, 1982). Prior to the official nomenclature designations in 1980, IFN- $\alpha$  and IFN- $\beta$  were termed leukocyte and fibroblast IFN respectively, since the predominant form of IFN produced by each of these cell types found to be antigenically distinct (Navell *et al.*, 1975; Edy *et al.*, 1976). These terms were however misleading and were abandoned since leukocytes and fibroblasts are able to produce both antigenic species of IFN (Dalton and Faucker, 1979; Pang *et al.*, 1980). Throughout this thesis IFN- $\alpha$  and IFN- $\beta$  will be referred to as IFN- $\alpha\beta$  whenever the exact type has not been specified or where a mixture of the two types exists.

DNA sequence analysis of molecularly cloned IFN- $\alpha$  has revealed that the antigenically distinct IFN- $\alpha$  species actually consist of a family of closely related proteins (designated IFN- $\alpha_1$ , IFN- $\alpha_2$ , etc ...) which are between 161 and 167 amino-acids long in the murine system, and between 165 and 172 amino-acids long in man (Kavada, 1987). The IFN- $\alpha$  proteins have around 80% homology in amino-acid sequence (Knight, 1984) and are coded for by a multi gene family comprised of at least 10 genes in the murine system, and at least 13 genes in man (Goeddel *et al.*, 1981; Shaw *et al.*, 1983; Kavada, 1987). In contrast, there is only one gene for IFN- $\beta$  in both the

murine and human system, which codes for a protein 161 and 166 amino-acids long, respectively (Derynck *et al.*, 1980; Higashi *et al.*, 1983). A second, putative IFN- $\beta_2$  gene has been cloned in both the murine and human system although it is at present unclear whether the protein coded has an antiviral activity (Marx, 1988). Both murine and human IFN- $\alpha$  and  $\beta$  genes do not contain introns (Nagata *et al.*, 1980; Degraeve *et al.*, 1981; Higashi *et al.*, 1983; Shaw *et al.*, 1983; Gray and Goeddel, 1983) and are located on chromosome 4 in the mouse (Kelley *et al.*, 1983) and on chromosome 9 in man (Shows *et al.*, 1982). DNA sequence analysis has revealed regions of extensive homology within the upstream flanking regions of the IFN- $\alpha$  and IFN- $\beta$  genes, which are not found associated with the IFN- $\gamma$  gene (Degraeve *et al.*, 1981; Gray and Goeddel, 1982). These regions may contain the consensus sequences regulating the expression of the IFN- $\alpha$  and IFN- $\beta$  genes, and may explain why these IFNs are often produced simultaneously (Riordan and Pitha-Rowe, 1983). Fujita and coworkers have recently isolated a virus-inducible gene encoding a transcription factor IRF-1 (interferon regulatory factor-1) which mediates the transcription of IFN- $\alpha$  and IFN- $\beta$  genes (Miyamoto *et al.*, 1988; Fujita *et al.*, 1989). IRF-1 has been shown to bind to the upstream regulatory region of the IFN- $\beta$  gene (Fujita *et al.*, 1988) but it is not yet known whether IRF-1 also binds to the homologous upstream region of the IFN- $\alpha$  genes. IFN- $\alpha$  and IFN- $\beta$  are related proteins which share 20% homology at the amino-acid level in the mouse (Dijkman and Billiau, 1985) and 29% homology in man (Taniguchi *et al.*, 1980). Both share a common receptor on the surface of cells which are sensitive to their effects (Aguet and Blanchard, 1981; Branca and Baglioni, 1981) and are generally stable at pH2 and 36°C, although an acid labile IFN- $\alpha$  has been reported (Preble *et al.*, 1982; Balkwill, 1983). IFN- $\gamma$ , which was first described in 1965 as being produced in lymphocyte cultures stimulated by the T-cell mitogen phytohemagglutinin (Wheeler, 1965), differs from

IFN- $\alpha$  and IFN- $\beta$  not only by its mode of induction but also by its physicochemical properties and genetic organisation. Thus, to date one IFN- $\gamma$  gene has been identified in both the murine and human system, and in contrast to the genes for IFN- $\alpha$  and IFN- $\beta$ , contains three introns (Gray and Goeddel, 1982, 1983) and is located on chromosome 12 in the mouse (Maylor *et al.*, 1984) and on chromosome 10 in man (Trent *et al.*, 1982). DNA sequence analysis of molecularly cloned IFN- $\gamma$  has also revealed the IFN- $\gamma$  molecule, which is 136 amino-acids long in the murine system (Gray and Goeddel, 1983) and 146 amino-acids long in man (Gray and Goeddel, 1982) to be shorter than both IFN- $\alpha$  and IFN- $\beta$ . Furthermore, the primary amino-acid sequence of IFN- $\gamma$  is unrelated to that of IFN- $\alpha$  and IFN- $\beta$  (Dijkman and Billiau, 1985). IFN- $\gamma$  is labile at pH 2 and 56°C, and binds to a receptor on the surface of cells which appears to be distinct from that used by IFN- $\alpha$  and IFN- $\beta$  (Branca and Baglioni, 1981; Aguet and Blanchard, 1981). It is now clear that IFN- $\beta$  and IFN- $\gamma$  are glycosylated in both the murine and human system (Bocci, 1983; Kawada, 1987). The subtypes of murine IFN- $\alpha$  are also glycosylated (Fujisawa *et al.*, 1978; Kawada, 1987) but in contrast the majority of human IFN- $\alpha$  subtypes are non-glycosylated with only 3 glycosylated subtypes recognised to date (Zoon *et al.*, 1987).

Glycosylation of IFN does not however appear to be important for biological activity since recombinant IFNs produced in bacteria (hence which are non-glycosylated) have activities comparable, at least *in vitro*, to those displayed by natural IFN (McCullagh *et al.*, 1983). The possibility that glycosylation may influence the stability and tissue specificity of IFNs *in vivo* has been suggested as a result of the observations that human IFN- $\beta$  and IFN- $\gamma$  when administered intramuscularly were found to remain at this site, whereas IFN- $\alpha$  when administered intramuscularly rapidly diffused into the plasma (Bocci, 1983).

Although IFNs were originally identified and are currently defined by virtue of their antiviral activity, it is now clear that they also exert a series of effects on immune cells, on myelomonocytic cells, and on a variety of other cell types (Trinchieri and Ferruscia, 1985; Vilcek and De Maeyer, 1984). Some of these effects are mediated by all three IFN types whilst others are mediated exclusively by IFN- $\gamma$ . In the cases where the effects are mediated by all three IFN types, IFN- $\gamma$  has been shown to be effective at significantly lower concentrations (relative to antiviral activity units) than IFN- $\alpha$  or IFN- $\beta$ . For example, Varesio and coworkers demonstrated that both natural IFN- $\beta$  and recombinant IFN- $\gamma$  activated murine macrophages for tumour cell killing *in vitro*, and showed that IFN- $\gamma$  was 100 to 1000 times more potent than IFN- $\beta$  in inducing this activity (Varesio *et al.*, 1984). Similar observations were also made by Pace *et al.* (1985) who found that recombinant IFN- $\gamma$  was 500 to 1000 times more active than both natural IFN- $\alpha$  and natural IFN- $\beta$  in inducing murine macrophage tumoricidal activity *in vitro*. Recombinant IFN- $\alpha$ , natural IFN- $\beta$  and recombinant IFN- $\gamma$  have also been shown to increase murine macrophage Fc receptor mediated phagocytic activity *in vitro*, and again IFN- $\gamma$  was found to be 10 to 1000 times more potent in this respect (Fertsch and Vogel, 1984). Rubin and Gupta (1980) compared the antiproliferative effect of human natural IFN- $\alpha\beta$  and natural IFN- $\gamma$  *in vitro*, and found that IFN- $\gamma$  was more than 160 times more active in inhibiting human fibroblast proliferation. Human recombinant IFN- $\gamma$  has also been shown to be 50 times more active than human natural IFN- $\alpha$  and IFN- $\beta$  in enhancing the natural killer activity of human lymphocytes *in vitro* (Weigent *et al.*, 1983). Studies by Wallach and coworkers also demonstrated that human natural IFN- $\gamma$  increased class I major histocompatibility complex (MHC) HLA-A, -B, -C, antigen expression on human lymphoblastoid cells at concentrations 100 times lower than needed to induce an antiviral state. This difference was not found with human

natural IFN- $\alpha$  or IFN- $\beta$  which increased class I MHC antigen expression and induced an antiviral state over the same range of concentrations (Wallach et al., 1982). Taken together the results of these studies clearly indicate that IFN- $\gamma$  is a potent immunomodulator and suggest that the antiviral activity may not be its most important function.

When protein extracts of human fibroblasts induced with human natural IFN- $\alpha$ , IFN- $\beta$  or IFN- $\gamma$  are analysed by two-dimensional gel electrophoresis, it is clear that all three IFN types induce the synthesis of a common group of polypeptides (Weil et al., 1983). These probably include those responsible for mediating the shared activities of IFNs. The synthesis of two proteins (molecular weights 56 and 67 kilodaltons) induced by both natural IFN- $\alpha$  and IFN- $\gamma$  has also been found to be regulated differently in human fibroblasts. Thus, synthesis of these proteins was regulated both at the transcriptional level and at the translational level in cells treated with IFN- $\alpha$ , whereas the translational regulation was inoperative in IFN- $\gamma$  treated cells (Sen and Rubin, 1984). Clearly, even in the shared activities of the two IFN types, the molecular mechanisms involved are not identical. IFN- $\gamma$  has also been shown to induce a unique set of twelve distinct polypeptides in addition to the common group of polypeptides induced by all three IFN types (Weil et al., 1983). This additional set of polypeptides may mediate the activities which appear to be exclusive to IFN- $\gamma$ , an example of which is the ability to increase (or induce) class II MHC antigen expression on a number of cell types (Trinchieri and Perussia, 1985).

As mentioned previously, it is now clear that the IFN- $\alpha$  species actually consist of a family of closely related proteins. The biological significance of this diversity may lie with the observations that a number of IFN- $\alpha$  subtypes differ in biological activity. For example, Evinger and

coworkers purified eight subtypes of human natural IFN- $\alpha$  and showed that the ratio of antiproliferative to antiviral activity was not constant for each of the subtypes but varied upto thirty fold. Furthermore, the ratio of antiviral activity on human cells to that on bovine cells differed between the IFN- $\alpha$  subtypes (Evinger *et al.*, 1981). Similar observations were also made by Streuli *et al.* (1980) who compared the antiviral activities of recombinant human IFN- $\alpha$ 1 and IFN- $\alpha$ 2 (bacterial products were used) on human and bovine cells, and showed that IFN- $\alpha$ 1 was 10-20 fold more active on bovine cells than on human cells while IFN- $\alpha$ 2 was twice as active on human cells as on bovine cells. Recombinant human IFN- $\alpha$ D has also been shown to be slightly more active than recombinant human IFN- $\alpha$ A in inducing the natural killer cell activity of human lymphocytes (both IFN- $\alpha$ A and IFN- $\alpha$ D were bacterial products, IFN- $\alpha$ A = IFN- $\alpha$ 2 and IFN- $\alpha$ D = IFN- $\alpha$ 1 : Taylor-Fapadimitriou, 1985) (Targan and Stebbing, 1982).

## (2) Mechanisms of the antiviral effect of IFN on cells

IFN has no direct effect on viral particles. Instead, all antiviral effects of IFN are the results of biochemical modifications of treated cells whereby the cells are rendered unable to proceed through the various steps required for the synthesis and assembly of virions.

As mentioned previously, IFN- $\gamma$  binds to a receptor on the surface of cells which appears to be distinct from that used by IFN- $\alpha$  and IFN- $\beta$  (Branca and Baglioni, 1981; Aguet and Blanchard, 1981). The presence of a specific receptor at the cell-surface is the first requirement for IFN action : cells which do not express receptors are resistant to the action of IFN (Aguet, 1980; Marti *et al.*, 1981). Cells respond to IFN treatment by initiating a series of biochemical changes which eventually result in the



development of an antiviral state. Not only is the activation of these biochemical changes different in different cell-types but viruses show a differential susceptibility to their action. For example, whilst encephalomyocarditis virus and vesicular stomatitis virus are very sensitive to the antiviral activity of IFNs, the replication of these two viruses is inhibited by different IFN-induced biochemical changes in a given cell-type (Taylor-Papadimitriou, 1985). In this section I shall briefly outline the mechanisms whereby IFNs may exert their antiviral effect. For a comprehensive review of this area the reader is referred to Friedman (1984).

The antiviral effect of IFN is considered to depend, at least in part, on the expression of two enzymic pathways : the 2',5'-oligoadenylate synthetase-nuclease pathway, and the elongation initiation factor-2 (eIF-2) protein kinase pathway. Both enzyme systems are induced by IFNs- $\alpha$ , - $\beta$  and - $\gamma$ , but remain in a latent state until they are activated by double-stranded ribonucleic acid (ds RNA) (Content, 1984). Experiments performed *in vitro* have shown that the 2',5'-oligoadenylate synthetase is activated when bound to ds RNA containing more than 30 base-pairs. ds RNA of this size is thought to be only found in virus-infected cells (Baglioni, 1987). Activated 2'-5' oligoadenylate synthetase has been shown to synthesise 2'-5' linked oligoadenylates [pppA(2'p5'A)n] (known as 2-5A) from adenosine triphosphate (Minks *et al.*, 1979). 2-5A activates endogenous ribonuclease L by binding to the enzyme. Activated ribonuclease L degrades viral messenger RNA and is thus thought to inhibit viral replication (Baglioni, 1987). A number of studies have indicated that the 2-5A system is operative in intact cells. Thus, Nilsen *et al.*, (1982a) showed that 2-5A was synthesised in reovirus-infected IFN- $\beta$  treated human HeLa cells and that viral messenger RNA was degraded. Similarly, in encephalomyocarditis

virus-infected IFN- $\alpha\beta$  treated murine L cells the 2-5A synthetase is activated, 2-5A is present (Knight *et al.*, 1980), and the ribonuclease is activated (Wreschner *et al.*, 1981). Chebath *et al.* (1987) transfected chinese hamster ovary cells with cDNA encoding the 2-5A synthetase enzyme. They obtained transfectants which expressed high constitutive levels of 2-5A synthetase and showed that the elevated enzyme activity correlated with resistance to infection by Mengovirus, but did not make the cells resistant to vesicular stomatitis virus. These observations clearly indicate that 2-5A synthetase is sufficient to protect chinese hamster ovary cells against infection by Mengovirus, but not by vesicular stomatitis virus. A 67-73 kilodalton ds RNA-dependant protein kinase has been detected in cellular extracts of IFN- $\alpha\beta$ -treated cells by several groups (Sen *et al.*, 1978; Baglioni and Maroney, 1980). Purified ds RNA-dependant protein kinase has been shown to phosphorylate the smallest subunit ( $\alpha$ ) of eIF-2 (Berry *et al.*, 1985). Phosphorylation of eIF-2 impairs its ability to participate in the formation of the 40S initiation complex, and thus leads to an inhibition of protein synthesis initiation (Jagus *et al.*, 1980). A number of studies have indicated that the protein kinase system is operative in intact cells. Thus, Gupta and Holmes (1982) detected kinase activation (i.e. 67 kd protein phosphorylation) in IFN- $\alpha\beta$  treated and reovirus-infected murine L929 cells. Nilsen *et al.* (1982b) showed that reovirus-infection of IFN- $\beta$  treated HeLa cells resulted in the elevation of kinase activity and the concomitant phosphorylation of eIF-2. Two HeLa lines which showed different sensitivities to IFN induced inhibition of reovirus growth had comparable levels of 2-5A synthetase activity, but the more sensitive line demonstrated 3-4 times more protein kinase activity and an increased phosphorylation of eIF-2 (Nilsen *et al.*, 1982b). A lack of correlation between the induction of protein kinase activity and inhibition of vesicular stomatitis virus replication in several murine cells has also

been reported (Sen and Merz, 1983). Clearly, as for the 2-5A system, the relative importance of protein kinase in mediating the antiviral effect of IFN is dependant on the virus-cell system being used.

The antiviral effects of IFNs cannot be accounted for solely by the 2-5A and protein kinase pathways. For example, Lewis (1988) showed that IFN- $\beta$  induced an antiviral state which prevented replication of vesicular stomatitis virus, Mengovirus and reovirus in murine Ltk cells in the absence of elevated levels of 2-5A synthetase and eIF-2 kinase. Several studies have shown that IFNs may also have a number of effects on the final maturation of virus. For example, in IFN treated Ocoronavirus-infected cells there is no detectable inhibition of viral RNA or protein synthesis but an accumulation at the cell-surface of non-infectious defective virus-particles (Friedman *et al.*, 1975; Pitha *et al.*, 1976; Billiau *et al.*, 1976). The mechanism(s) whereby these IFN-induced abnormalities in assembly, maturation and release occur are not known. Maheshwar *et al.* (1980) showed that whilst low amounts of IFN- $\alpha\beta$  reduced the yield of vesicular stomatitis virus particles from murine L cells 10-fold, the infectivity of progeny particles was reduced 1000-fold. Subsequently, the reduction in infectivity was shown to result from the specific inhibition of incorporation of the G and M proteins into maturing virions in IFN-treated cells (Jay *et al.*, 1983).

Alleles of a mouse gene, Mx, have been shown to selectively influence the ability of IFN- $\alpha\beta$  (but not IFN- $\gamma$ ) to protect cells against influenza virus infection. Thus, cells derived from mice carrying the Mx gene are protected against influenza virus infection by much lower concentrations of IFN- $\alpha\beta$  than cells from non-Mx bearing strains (Taylor-Papadimitriou, 1985). The kinetics of protection against influenza in Mx-bearing peritoneal

macrophages has been shown to be markedly different from the antiviral state induced against vesicular stomatitis virus in these cells, indicating that the two antiviral mechanisms are unrelated (Arnheiter and Haller, 1983). A 72 kilodalton protein which is specific to Mx-bearing cells has been described, and an mRNA specific to IFN- $\alpha\beta$  treated Mx-carrying cells has been isolated which codes for a similar protein (Horisberger *et al.*, 1983; Staeheli *et al.*, 1983; Taylor-Papadimitriou, 1985). This protein is not induced by IFN- $\gamma$  (Taylor-Papadimitriou, 1985). The role of this protein in mediating the antiviral effect of IFN- $\alpha\beta$  in Mx-bearing cells is not known but there is a marked inhibition of translation of influenza mRNA in these cells with other steps remaining unaltered (Meyer and Horisberger, 1984).

### (3) Importance of major histocompatibility complex antigens in the immune system

#### (a) Introduction to the major histocompatibility complex antigens

The major histocompatibility complex (MHC) is a highly polymorphic region of the genome whose products play a major role in the immune response. It is known as the MHC for historical reasons since the molecules of this complex were first recognised as classical transplantation antigens on the cell-surface. The antigens were initially characterised in the murine system using alloantibodies, produced in one inbred strain of mice immunised with cells of other strains differing only at the MHC. Subsequently, with the use of specific antibodies to molecules encoded by small regions of the MHC and using techniques of protein chemistry and molecular biology, the characteristics of MHC genes and their products have been analysed in great detail.

The MHC in man, known as the human leukocyte antigen (HLA) system, is coded for by a genetic region located on the short arm of chromosome 6. In the murine system the MHC has been mapped to a genetic region (termed H-2) on chromosome 17 (McConnell *et al.*, 1984). Three classes of molecules, denoted I, II and III, have been identified in the MHC of both mouse and man. At least three separate class I loci (termed H-2K, -D and -L in the mouse and HLA-A, -B and -C in man) encoding classical transplantation antigens have been demonstrated. Other class I genes in the mouse map at the right of the MHC in regions known as Qa-2,3 and Tla (Male *et al.*, 1987). The class I MHC genes are highly polymorphic. Thus, more than 50 different alleles have been demonstrated at both the H-2K and H-2D class I loci of mice. In contrast, the Qa-2,3 and Tla region class I genes exhibit little polymorphism (Male *et al.*, 1987). The class I MHC antigens are comprised of a glycosylated polypeptide chain of 45 kilodaltons (Kd) (heavy chain) in close non-covalent association with  $\beta_2$ -microglobulin, a 12 Kd polypeptide (Law *et al.*, 1986).  $\beta_2$ -microglobulin is encoded outside the MHC on human chromosome 15 (Goodfellow *et al.*, 1975) and on mouse chromosome 2, and appears to stabilise the class I MHC molecule (Male *et al.*, 1987). Amino-acid sequence analyses of human and murine class I molecules have demonstrated that the heavy chain is divided into five distinct regions: three extracellular domains of about 90 amino-acids designated  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$ , a transmembrane region of about 25 hydrophobic amino-acids, and a cytoplasmic domain which consists of about 25 hydrophobic amino-acids. Interchain disulphide bonds in the  $\alpha_2$ ,  $\alpha_3$  and  $\beta_2$ -microglobulin domains stabilise the structure of the molecule (see figure 1.1a). Very recently, Bjorkman *et al.* (1987a, 1987b) determined the three-dimensional structure of the human class I MHC HLA-A2 antigen by X-ray crystallography, and confirmed the presence of the  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and  $\beta_2$ -microglobulin domains (see figure 1.1b). Class I MHC antigens can be

Figure 1.1a

Structure of the murine class I MHC H-2K<sup>b</sup> antigen

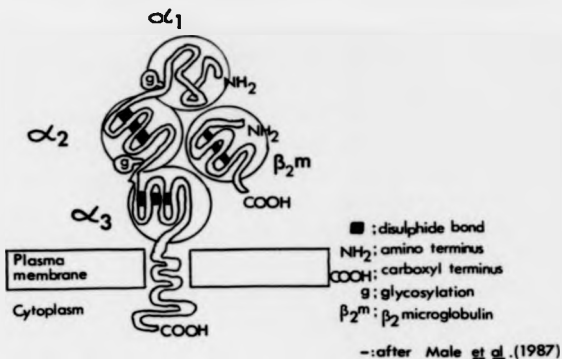
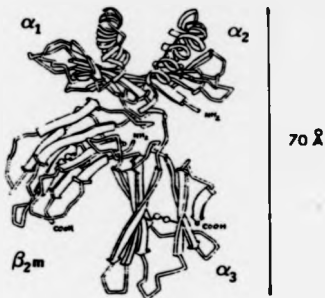


Figure 1.1b

Three-dimensional structure of human class I MHC HLA-A2 antigen



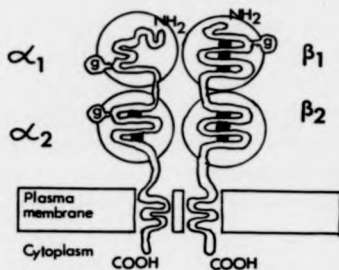
--after Bjorkman *et al.* (1987)

detected by indirect immunoperoxidase staining on the surface membrane of virtually all nucleated cells (exceptions being hepatocytes and cells of the central nervous system, exocrine pancreas and cornea-endothelium : Male *et al.*, 1987) and are intimately involved in the presentation of antigen to cytotoxic T-cells (see section c below). The products of the Qa-2,3 and Tla loci are 44 Kd proteins which are both found associated with  $\beta_2$ -microglobulin (McConnell *et al.*, 1984). Qa-2,3 and Tla proteins have been found on thymocytes, and Qa-2 protein on some peripheral T-cells (McConnell *et al.*, 1984). Whether these proteins are normally expressed on other cell-types and whether they are involved in the presentation of antigen to cytotoxic T-cells is still unknown (Male *et al.*, 1987). It has been suggested that the Qa-2,3 and Tla region genes provide a pool of genetic information which is used in the generation of polymorphism of class I molecules (Male *et al.*, 1987).

The highly polymorphic class II genes, encoded in the H-2I region of the mouse MHC and the HLA-D region of man, are identical to the immune response (I<sub>r</sub>) genes known to control murine responses to different antigens (e.g. to thyroglobulin, for review see McConnell *et al.*, 1984). The products of class II genes (I-A and I-E in the mouse, DP, DQ and DR in man) are heterodimers comprising heavy ( $\alpha$ ) and light ( $\beta$ ) glycoprotein chains. The  $\alpha$  chains have molecular weights of 30 to 34 Kd and the  $\beta$  chains range from 26-29 Kd, depending on the alleles involved (Male *et al.*, 1987). On the basis of amino-acid sequence, each chain has been shown to consist of four domains: two extracellular domains of about 90 amino-acids designated  $\alpha_1$  and  $\alpha_2$  or  $\beta_1$  and  $\beta_2$ , a transmembrane region of about 30 amino-acids, and a short cytoplasmic domain of between 10 and 15 hydrophobic amino-acids (Male *et al.*, 1987). The  $\alpha_2$ ,  $\beta_1$  and  $\beta_2$  domains contain disulphide bonds which stabilise the molecule (see figure 1.2). The cellular distribution of

Figure 1.2

Structure of class II MHC antigens



- : disulphide bond
- $\text{NH}_2$  : amino terminus
- $\text{COOH}$  : carboxyl terminus
- g : glycosylation
- $\beta_2^m$  :  $\beta_2$  microglobulin

—after Male et al. (1987)



class II MHC antigens (also known as immune-associated [Ia] antigens) is not as wide as for class I MHC antigens. Only a few cell types constitutively express class II MHC antigens (for example, B-cells and dendritic cells; Knight et al., 1987; Male et al., 1987) although a number of other cell-types express class II MHC antigens when induced by treatment with IFN- $\gamma$  (for example, macrophages, astrocytes and fibroblasts - see section b below).

The class III genes encode the C<sub>2</sub>, C<sub>4</sub> and factor B components of the complement system (McConnell et al., 1984). There is no evidence for functional or structural similarities between these products and the class I and class II antigens, hence these genes may be better considered as being closely linked to the MHC rather than part of it (Male et al., 1987).

#### (b) Interferon modulation of MHC antigen expression

In 1973 Lindahl and coworkers demonstrated that natural IFN- $\alpha\beta$  treated mouse lymphoma L1210 cells (H-2<sup>d</sup>) displayed an enhanced antibody absorbing capacity for serum from C57 BL/6 mice (H-2<sup>b</sup>) immunised with Balb/c (H-2<sup>d</sup>) splenic lymphocytes (Lindahl et al., 1973). Although the antiserum was crude, these observations suggested that IFN- $\alpha\beta$  increased MHC antigen display on the surface of L1210 cells. Subsequently, using monospecific antisera against determinants controlled by subregions of the H-2 complex, it was shown that natural murine IFN- $\alpha\beta$  enhanced class I MHC H-2D and H-2E antigen expression, but not class II MHC antigen expression, on mouse splenic lymphocytes in vitro (Lonsi and Steinman, 1977; Vignaux and Gresser, 1977), on mouse embryo fibroblasts in vitro (Vignaux and Gresser, 1978), and on mouse splenic lymphocytes in vivo (Vignaux and Gresser, 1977). Several years later Sonnenfeld and coworkers demonstrated that

crude preparations of natural IFN- $\gamma$  (prepared by pulsing spleen cells of Swiss/Webster mice sensitised to *Bacillus Calmette-Guérin* with tuberculin in vitro) increased both class I and class II MHC antigen expression on murine thymocytes in vitro (Sonnenfeld et al., 1981).

Since this time IFNs have been shown to modulate MHC antigen expression on a wide variety of cell types in vitro in both the murine and human system. With few exceptions, IFN- $\alpha\beta$  have been shown to induce only class I MHC antigen expression whilst IFN- $\gamma$  induces both class I and class II (on certain cell types only) MHC antigen expression. For example, IFN- $\alpha\beta$  have been shown to induce class I, but not class II, MHC antigen expression on human lymphoblastoid cells (Fellous et al., 1979), human melanoma cells (Imai et al., 1981), human keratinocytes (Niedervieser et al., 1988), murine fibroblasts (Dr A Morris, University of Warwick, unpublished observations), and murine astrocytes (Wong et al., 1985). In contrast, IFN- $\gamma$  has been shown to induce both class I and class II MHC antigen expression on murine B cells, macrophages and mast cells (Wong et al., 1984c; Koch et al., 1984), murine fibroblasts (Maudsley and Morris, 1988), human keratinocytes (Niedervieser et al., 1988), murine astrocytes (Wong et al., 1985), and human melanoma cells (Rosa et al., 1983b). IFN- $\gamma$  has also been shown to induce class II MHC antigen expression on human monocytes (Virelizier et al., 1984), human endothelial cells (Manyak et al., 1988), human glioma cells (Takiguchi et al., 1985), rat Schwann cells (Wekerle et al., 1986a), and murine microglial cells (Suzumura et al., 1987), but does not induce class II MHC antigen expression on human T-cell lines (Gerrard et al., 1988), human neuroblastoma cells (Lampson and Fischer, 1984), and murine oligodendrocytes (Suzumura et al., 1986). In exception to the general trend, IFN- $\alpha_1$  (but not IFN- $\alpha_2$ ) has been shown to induce class II MHC antigen expression on human monocytes (Rhodes et al., 1986).

Similarly, IFN- $\alpha$  and IFN- $\beta$  have been shown to induce class II MHC antigen expression on human melanoma cells (Dolei *et al.*, 1983). In many of the above studies preparations of recombinant IFNs were used, thereby confirming that MHC antigen expression was indeed modulated by IFN rather by contaminants which may be present in natural IFN preparations. IFNs have also been shown to induce MHC antigen expression on a variety of cell types *in vivo*. Thus, Momburg *et al.* (1986a) demonstrated that intravenous administration of recombinant IFN- $\gamma$  to B10.BR mice induced or enhanced class I MHC antigen expression in almost every organ, and also reported that the effect was particularly conspicuous on renal tubular cells, hepatocytes, bronchiolar epithelial cells, gastric mucous cells, thymic cortical lymphocytes and capillary endothelial cells in the heart and kidney. Intravenous and intraperitoneal administration of recombinant IFN- $\gamma$  to B10.BR mice has also been shown to increase class II MHC antigen expression throughout the body, with marked increases observed in the heart, kidney, pancreas, lung, liver, adrenal, and small intestine (Skoekiewicz *et al.*, 1985; Momburg *et al.*, 1986b). Intravenous administration of recombinant IFN- $\gamma$  to B10.BR mice did not induce class I or class II MHC antigen expression on neurons or glial cells within the brain (Momburg *et al.*, 1986a, 1986b). In contrast, intracerebral administration of recombinant IFN- $\gamma$  to CBA mice has been shown to induce class I MHC antigen expression on neurons and glial cells, and class II MHC antigen expression on astrocytes (a glial cell) (Wong *et al.*, 1984). Taken together, these observations indicate that IFN- $\gamma$  does not normally traverse the blood-brain barrier of a healthy mouse. Intraperitoneal administration of recombinant IFN- $\gamma$  to C3H/HeN mice has also been shown to induce class II MHC antigen expression on keratinocytes (Gaspari and Katz, 1988). Furthermore, intravenous administration of natural IFN- $\beta$  to Balb/c mice has been shown to induce class I MHC antigen expression on bone marrow cells

(Bukowski and Walsh, 1986).

Wong *et al.*, (1984) showed that actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (an inhibitor of protein synthesis) blocked the induction of class I MHC antigens on murine brain cells *in vitro*, indicating that the induction required new RNA and protein synthesis. Similarly, Rosa *et al.* (1983b) reported that the enhancement of class I MHC antigen expression on human lymphoid cells *in vitro* was preceded by an increase in class I MHC protein synthesis. Northern blot analysis of mRNA encoding class I and class II MHC antigens has shown that IFN- $\alpha\beta$  increases the steady state level of class I MHC mRNA in human fibroblasts and lymphoblastoid cells (Fellous *et al.*, 1982). Likewise, IFN- $\gamma$  has been shown to increase the steady state level of class I MHC and  $\beta_2$ -microglobulin mRNA in human lymphoblastoid cells (Wallach *et al.*, 1982), and class II mRNA in human glioma and macrophage cell lines (Takiguchi *et al.*, 1986; Nakamura *et al.*, 1984). The steady state level of mRNA in a cell is the result of several processes : transcription of the gene, processing of the primary nuclear transcript, and mRNA degradation within the cell (Rosa *et al.*, 1986). The increased level of mRNA encoding class I and class II MHC antigens in IFN treated cells may thereby reflect modifications of transcription, processing or degradation of the mRNA. To determine whether IFN- $\beta$  and IFN- $\gamma$  increased transcription of class I, class II MHC and  $\beta_2$ -microglobulin genes, Rosa *et al.* (1986) followed the transcription of the genes in isolated nuclei using the "nuclear run-off" technique. The nuclear run-off technique is based on the fact that RNA polymerases in isolated nuclei are able to carry out elongation of the nascent RNA chain, but cannot promote the initiation of new mRNA molecules. Thus, incorporation of radiolabelled nucleotides into RNA, which is able to hybridize to probes for the genes of interest, allows one to quantify the

rate of transcription of the genes. Using this technique, Rosa *et al.* (1986) showed that IFN- $\beta$  and IFN- $\gamma$  induced an increase in the transcription of class I MHC and  $\beta_2$ -microglobulin genes in human melanoma cells, and that IFN- $\gamma$  (but not IFN- $\beta$ ) induced transcription of class II MHC genes in human fibroblasts.

Friedman and Stark (1985) compared the DNA sequences of four human genes which are sensitive to IFN regulation: two class I MHC genes, a class II MHC gene, and a metallothionein gene. They defined a consensus sequence 30 nucleotides long in the promoter region of each gene which was postulated to be involved in regulating transcription. The promoters of several murine class I MHC genes have also been shown to contain a similar sequence (Israel *et al.*, 1986). Israel *et al.* (1986) showed that the murine class I MHC H-2K<sup>b</sup> gene promoter could be induced by IFNs - $\alpha$ , - $\beta$  and - $\gamma$ , and that the consensus sequence was necessary for induction to occur. Another consensus sequence has recently been found in the promoter region of several IFN- $\gamma$ -inducible human and murine class I and class II MHC genes, although its efficacy has not yet been shown (Basta *et al.*, 1987). It has been speculated that there may be separate and distinct regulatory regions for the different classes of IFN (Basta *et al.*, 1987).

Tumour necrosis factor (TNF- $\alpha$ ) and lymphotoxin (now known as tumour necrosis factor- $\beta$  [TNF- $\beta$ ]) have also been shown to increase class I, but not class II, MHC antigen expression on human endothelial cells *in vitro* (Laevenberg *et al.*, 1987; Lapiere *et al.*, 1988; Wedgewood *et al.*, 1988), and, for the case of TNF- $\alpha$ , class I MHC antigen expression on human fibroblasts *in vitro* (May *et al.*, 1986; Laevenberg *et al.*, 1987), and both class I and class II MHC antigen expression on the murine WEHI-3 macrophage cell-line (Chang and Lee, 1986). The observations that TNF- $\alpha$  induced FS4

fibroblasts to produce IFN- $\beta_2$  mRNA (but not IFN- $\beta_1$  mRNA) (Kohase *et al.*, 1986; May *et al.*, 1986), and that cross-reacting polyclonal antiserum to IFN- $\beta$  inhibited the ability of TNF- $\alpha$  to induce class I MHC antigen expression on FS4 fibroblasts (May *et al.*, 1986), suggest that the effect of TNF- $\alpha$  on class I MHC antigen expression may actually be mediated by IFN- $\beta_2$ . However, Laevenberg *et al.* (1987) reported that the ability of TNF- $\alpha$  to induce class I MHC antigen on primary cultures of fibroblasts and endothelial cells was inhibited by antibodies which specifically neutralised IFN- $\beta_1$  (but not IFN- $\beta_2$ ). This apparent contradiction of results may be due to an ability of TNF- $\alpha$  to induce different cells to release different types of IFN- $\beta$ , or may be due to the fact that Kohase *et al.* (1986) and May *et al.* (1986) did not look specifically for IFN- $\beta_1$  protein. Nevertheless, it is clear from these studies that IFN- $\beta$  is an intermediate in the TNF- $\alpha$ -induced increase in class I MHC antigen expression.

TNF- $\alpha$  and - $\beta$  have also been shown to interact with IFN- $\gamma$  in the induction of MHC antigen expression on cells *in vitro*. Thus, Pujol-Borrell *et al.* (1987) showed that human pancreatic islet cells did not express class II MHC antigens when induced with TNF- $\alpha$ , TNF- $\beta$  or IFN- $\gamma$  alone, but expressed high levels when induced with IFN- $\gamma$  in combination with either TNF- $\alpha$  or TNF- $\beta$ . Weetman and Rees (1988) showed that IFN- $\gamma$  induced class II MHC antigen expression on rat thyroid cells (whereas TNF- $\alpha$  did not), and that TNF- $\alpha$  increased the ability of IFN- $\gamma$  to increase class II MHC antigen expression. Similarly, Wedgewood *et al.* (1988) showed that TNF- $\alpha$  slightly increased the ability of IFN- $\gamma$  to induce class II MHC HLA-DQ antigen expression on human endothelial cells, but slightly inhibited the ability of IFN- $\gamma$  to induce class II MHC HLA-DR antigen expression. In contrast, Lapiere *et al.* (1988) reported that TNF- $\alpha$  and TNF- $\beta$  did not affect the

ability of IFN- $\gamma$  to induce class II MHC HLA-DR antigen expression on human endothelial cells. Wedgewood *et al.* (1988) and Lapierre *et al.* (1988) both showed that TNF- $\alpha$  in combination with IFN- $\gamma$  induced class I MHC antigen expression on human endothelial cells to a level higher than was observed with either mediator alone. Taken together, the results of these studies suggest that TNF can modulate or act in synergy with IFN- $\gamma$  in the induction of class I and class II MHC antigen expression on a number of cell types, although details remain to be fully clarified.

The different IFN classes have also been shown to interact in the induction of MHC antigen expression. Thus, IFN- $\alpha$  and IFN- $\beta$  have an additive effect with IFN- $\gamma$  in the induction of class I MHC antigen expression on human endothelial cells (Lapierre *et al.*, 1988). In contrast, IFN- $\alpha$  and IFN- $\beta$  have been shown to inhibit the ability of IFN- $\gamma$  to induce class II MHC antigen expression on murine macrophages (Ling *et al.*, 1985; Inaba *et al.*, 1986; Fertsch *et al.*, 1987; Kitaura *et al.*, 1988), and on human endothelial cells (Lapierre *et al.*, 1988; Manyak *et al.*, 1988). Similarly, prostaglandin E has been shown to inhibit the ability of IFN- $\gamma$  to induce class II MHC antigen expression on murine macrophages, but not on human macrophages (Snyder *et al.*, 1982; Kunkel *et al.*, 1986). Corticosteroids, transforming growth factor- $\beta_1$ , transforming growth factor- $\beta_2$ , and interleukin-3 have also been shown to inhibit the ability of IFN- $\gamma$  to induce class II MHC antigen expression on murine macrophages, human melanoma cells, human glioma cells, and murine mast cells, respectively (Snyder and Unanue, 1982; Czarniecki *et al.*, 1988; Zuber *et al.*, 1988; Wong *et al.*, 1984b).

It is now clear that viruses may also modulate host cell MHC antigen expression *in vitro*, and by a mechanism which does not involve the

intervention of IFNs. For example, measles virus, JHM coronavirus, and simian immunodeficiency virus have been shown to induce class II MHC antigen expression on their host cell, and in the case of measles virus also class I MHC antigen expression (Massa *et al.*, 1987a; Massa *et al.*, 1986; Kannagi *et al.*, 1987). In contrast, adenovirus-12, herpes simplex virus types 1 and 2, hepatitis B virus, and Moloney and Kirsten murine sarcoma viruses have been shown to reduce class I MHC antigen expression on their host cell (Schrier *et al.*, 1983; Jennings *et al.*, 1985; Onji *et al.*, 1987; Flyer *et al.*, 1985; Maudsley and Morris, 1988). Hepatitis B virus and Kirsten murine sarcoma virus have also been shown to inhibit the ability of IFN- $\gamma$  to induce class I MHC antigen expression on their host cell, and for the case of Kirsten murine sarcoma virus also class II MHC antigen expression (Onji *et al.*, 1987; Maudsley and Morris, 1988). Since class I and class II MHC antigens are intimately involved in T-cell recognition of antigen (see sections c and d below), it has been proposed that virus inhibition of host cell MHC antigen expression may help the virus evade T-cell mediated immune surveillance. Virus induction of host cell class II MHC antigen expression has also been proposed to play a role in the generation of autoimmune disease. Thus, induction of class II MHC antigens may result in the generation of an inappropriate immune response to an antigen not normally seen by the immune system, for example, to myelin basic protein which results in allergic encephalomyelitis in rats (Massa *et al.*, 1987a, 1987b). For a comprehensive review of virus modulation of host cell MHC antigen display see Maudsley *et al.* (1989).

In summary, it is clear that IFNs are able to influence MHC antigen expression on a variety of cell types both *in vitro* and *in vivo*, and that other factors modulate the ability of IFNs to induce MHC antigen expression, at least *in vitro*. Although many effects observed *in vitro*



still remain to be extended to the in vivo situation, it does at least seem likely that the induction of MHC antigen expression in vivo during infectious or autoimmune disease may be mediated by a number of different factors which inhibit or act in synergy with IFN.

(c) Importance of class I MHC antigens for cytotoxic T-lymphocyte recognition of target cells

The fundamental importance of the MHC in T-cell mediated immune responses was first fully appreciated in 1974 with the studies of Zinkernagel and Doherty. Zinkernagel and Doherty (1974) showed that cytotoxic T-cells from mice infected with lymphocytic choriomeningitis virus (LCMV) could kill LCMV-infected target cells only if the targets possessed the same set of genetic determinants located at the H-2 complex (i.e. were of the same haplotype). This phenomenon was termed MHC restriction. Subsequently, the requirements for MHC matching were mapped to the K and D regions of the H-2 by the use of H-2 recombinant mice (Zinkernagel and Doherty, 1975; Gardner et al., 1975; Blanden et al., 1975; Doherty et al., 1976; for review see Zinkernagel and Doherty, 1979). McMichael (1978) extended these observations to the human system and showed that influenza-specific CTL only killed infected target cells that shared identical HLA-A and HLA-B locus antigens.

That the expression of class I MHC antigens is essential for CTL recognition of antigen was initially demonstrated using cell lines which do not express these antigens, an example being the murine F9 teratoma cell line. Zinkernagel and Oldstone (1976) showed that F9 cells do not express class I MHC antigens detectable by either serological or alloreactive CTL assays. When infected with either LCMV or vaccinia virus F9 cells were

found to express viral antigens on the cell surface and released infectious virus in amounts equivalent to class I antigen bearing cell lines. Despite these cells expressing sufficient viral antigen adequate for lysis by antiviral antibody plus complement or antibody-dependant cell-mediated cytotoxicity, these cells were not susceptible to lysis by virus-specific T-cells.

Similarly, Forman and Vitetta (1975) demonstrated that haptenated (trinitrophenyl-modified (TNP)) F9 cells were not susceptible to lysis by TNP-specific CTL unlike TNP modified class I MHC antigen expressing targets from the same strain of mouse from which the F9 cell line originated. The conclusion made from each of these experiments was the same, that the F9 cells lack MHC antigens which are required for CTL recognition. Studies have also related levels of MHC antigen expression on target cells with recognition by CTL. Thus, Kuppers *et al.* (1981) treated murine target cells with papain, prior to TNP modification, and then examined the susceptibility of the cells to lysis by both allogeneic and syngeneic CTL. Papain treatment removes MHC antigens from the surface of target cells, thereby allowing comparison between levels of MHC antigen on the target cell and lysis by CTL. It was found that papain treatment reduced the susceptibility of the target cells to lysis by TNP-specific CTL. Furthermore, the decrease in susceptibility followed the same dose and time kinetics as the reduction of MHC antigen expressed on the cell surface as assessed by serological assay and susceptibility to lysis by alloreactive CTL. These authors also used a panel of murine cell lines, known to display different levels of class I MHC antigen in order to study further the relationship between levels of MHC antigen expressed on the target cells and susceptibility to CTL lysis. As before, cells were TNP-modified and the susceptibility to lysis by alloreactive and TNP-specific CTL

measured. The results of these studies again showed that there was a direct correlation between the level of MHC antigen expressed on each cell type, as assessed by serological assay and susceptibility to alloreactive CTL lysis, and its susceptibility to TNP-specific CTL lysis.

In view of the ability of IFNs  $\alpha$ ,  $\beta$  and  $\gamma$  to augment the expression of class I MHC antigens, and the correlation between levels of class I MHC antigen expressed on the surface of target cells and susceptibility to CTL lysis, it is not unreasonable to propose that the enhancement of class I MHC antigens by IFN should make cells more susceptible to CTL lysis. This does seem to be the case since IFNs ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) have been shown to increase the susceptibility of the following cell types to alloreactive CTL lysis; murine C3H10T $\frac{1}{2}$  fibroblast and RDM4 lymphoblastoid cells (Blackman and Norris, 1985), murine bone marrow cells and fibroblasts derived from heart, brain, liver, and skin (Bukowski and Walsh, 1986), and rat mammary carcinoma cells (Yeoman and Robbins, 1988). As mentioned previously, transformation of murine cells by adenovirus-12 reduces the level of class I MHC antigen on the cell-surface. Transformation by adenovirus-12 also reduces the susceptibility of cells to both alloreactive and influenza-specific CTL lysis (Eager *et al.*, 1985; Yawdell *et al.*, 1988a). Likewise, Moloney murine sarcoma virus inhibits the ability of Moloney murine leukemia virus to induce class I MHC antigen expression on murine fibroblasts, and inhibits the ability of Moloney murine leukemia virus to augment the susceptibility of infected cells to alloreactive CTL lysis (Flyer *et al.*, 1985). IFN- $\gamma$  has been shown to increase class I MHC antigen expression on cells infected with Moloney murine sarcoma virus, and on cells transformed by adenovirus-12, and increases the susceptibility of the cells to alloreactive CTL lysis (Eager *et al.*, 1985; Flyer *et al.*, 1985). Probably the strongest evidence to date which suggests that the level of

class I MHC antigen expression of target cells is important for T-cell recognition *in vivo* comes from the experiments of Bernard *et al.* (1983). These studies demonstrated that whereas both adenovirus-5 transformed cells (Ad-5; which express class I MHC antigens) and adenovirus-12 transformed cells (Ad12; which do not express class I MHC antigens) were oncogenic in T-cell deficient nude mice, only the class I MHC deficient Ad-12 transformed cells metastasised in normal mice. These results indicate that T-cells can play a role in the regulation of tumour growth, but only if the transformed cells express the class I MHC antigens necessary for T-cell recognition to occur. Likewise, the restoration of class I MHC antigen expression by gene transfection into MHC antigen-negative tumour cell clones has been found to reduce tumourigenicity and abolish metastasis in mice (Wallich *et al.* 1985). Furthermore, Hayashi *et al.* (1983) showed that Ad-12 transformed cells which were treated with IFN- $\gamma$ , and hence expressed class I MHC antigen, were less tumourigenic in mice than non-treated cells. Similarly, tumourigenicity of murine B16 melanoma cells on which class I MHC antigen expression had been induced by IFN- $\gamma$  was reduced compared with non-IFN treated cells (Hammerling *et al.* 1987). Although these data suggest that the reduced tumourigenicity of the cells may be due to increased expression of MHC antigens, it cannot be concluded that this is the only reason, since IFN- $\gamma$  also has effects on adhesion molecule expression and cell growth (Dustin *et al.* 1988; Balkwill, 1985).

Several studies have also investigated the effects of IFN-enhanced class I MHC antigen expression on virus-infected cells upon their susceptibility to virus-specific CTL lysis. This situation is more complex because whilst IFN enhances class I MHC antigen expression, it will generally inhibit virus replication and thus depress viral antigen expression, the other component essential for virus-specific CTL recognition. However, it has

been shown that even where viral antigen expression is markedly reduced by IFN treatment the level of virus-specific CTL lysis may only be slightly depressed or even augmented. For example, Blackman and Morris (1985) showed that treatment of murine EDMA lymphoblastoid cells and C3H10T $\frac{1}{2}$  fibroblasts with IFN- $\alpha\beta$  or - $\gamma$ , prior to infection with the neurotropic Semliki Forest virus (SFV), markedly increased the expression of class I MHC antigens on the cells but reduced the expression of viral antigen as assessed by antiviral antibody plus complement lysis. Despite the reduction of viral antigen expression cells remained susceptible to SFV-specific CTL lysis, presumably reflecting the enhancement of class I MHC antigen expression. Similar observations have also been made in other virus systems and these are discussed in detail in chapter 5.

The T-cell antigen receptor is a complex of the T1 molecule and T3 molecular complex. T1 is a highly polymorphic glycoprotein which consists of a disulphide-linked heterodimer formed of two peptides, known as the  $\alpha$  and  $\beta$  chains, which have molecular weights of 44 Kd and 37 Kd, respectively. The  $\alpha$  and  $\beta$  chains both consist of variable and constant domains (stabilised by intrachain disulphide bonds), a transmembrane region, and a cytoplasmic region (Samelson *et al.*, 1985; Male *et al.*, 1987). The T3 complex consists of four non-covalently associated peptides, known as  $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$  chains, which have molecular weights of 25 Kd, 21 Kd, 26 Kd and 16 Kd, respectively. The  $\gamma$  and  $\delta$  chains are glycosylated whereas the  $\epsilon$  and  $\zeta$  chains are not. The  $\epsilon$  chain possesses an intrachain disulphide bond and  $\zeta$  exists as a disulphide-linked homodimer. All four peptides are thought to traverse the cell membrane and possess a cytoplasmic region (Samelson *et al.*, 1985).

It is now clear that CTL use the  $\alpha$  and  $\beta$  chains of their T-cell receptor to

recognise specific antigen in association with class I MHC antigen on the target cell. The lack of direct involvement of other molecules (for example, those of the T3 molecular complex) in the specific recognition of antigen has been shown by transfection experiments. Thus, Dambic *et al.* (1986) transfected the  $\alpha$  and  $\beta$  chain genes of a fluorocelain (FL)-specific H-2D<sup>d</sup>-restricted CTL clone into a cytotoxic T-cell hybridoma specific for the hapten SF (3-[p-sulphophenyldiazo]-4-hydroxy-phenylacetic acid) in association with H-2K<sup>k</sup>, and obtained transfectants which could lyse FL-coupled H-2D<sup>d</sup> targets, as well as SF-coupled H-2K<sup>k</sup> cells. The activation of T-cells for both initiation of cytolytic activity and proliferation is dependant on an influx of Ca<sup>++</sup> ions. It has been proposed that the T3 molecular complex may act as an ion channel to effect the passage of Ca<sup>++</sup> across the membrane (Male *et al.*, 1987).

Townsend and coworkers studied antigen recognition by murine class I MHC restricted CTL clones specific for the nucleoprotein (NP) of influenza virus. They showed that the CTL were able to recognise and lyse L cells cotransfected with the NP gene and the appropriate class I gene. Furthermore, through the use of deletion mutants and synthetic peptides, short peptide regions of NP were identified as being recognised by the CTL (Townsend *et al.*, 1985; 1986). These observations suggest that target cells may have the potential to degrade viral proteins into peptide fragments, which are then presented in association with class I MHC antigens on the cell-surface and recognised by CTL. X-ray crystallographic studies by Bjorkman *et al.* (1987a, 1987b) revealed a deep groove on the top of the human class I MHC HLA-A2 antigen. The groove was reported to run between two long  $\alpha$ -helices derived from the  $\alpha 1$  and  $\alpha 2$  domains of the molecule, and the floor of the groove was formed by  $\beta$ -strands also derived from both  $\alpha 1$  and  $\alpha 2$  domains. Since the size of the groove was large enough

to accommodate peptides of 8 to 20 amino-acids in length (which is of the same order as the peptides used by Townsend) it was proposed that this may be the site for the foreign antigen that is recognised together with HLA antigen by the T-cell receptor. Very recently, Chen and Parham (1989) demonstrated that a 14 amino-acid fragment of influenza matrix protein did indeed directly bind to the human class I MHC HLA-A2 antigen. It has been suggested that the insertion of foreign peptide antigen into the groove of class I MHC antigens may take place within the cell and possibly as the class I molecules fold and associate with  $\beta_2$ -microglobulin (Townsend and McMichael, 1987).

Several groups have reported the isolation of class II MHC restricted CTL clones (Morrison *et al.*, 1986; Jones *et al.*, 1987). Class II MHC restricted CTL however make up only a minor proportion of the total CTL population, the majority being class I MHC restricted (Braakman *et al.*, 1987). Class II MHC restricted CTL are also thought to utilise the  $\alpha$  and  $\beta$  chains of their T-cell receptor to recognise specific antigen in association with the appropriate class II MHC antigen on the target cell (Male *et al.*, 1987). Morrison *et al.* (1986) studied antigen recognition by murine class II restricted CTL clones specific for haemagglutinin of influenza virus. They showed that isolated influenza virus haemagglutinin (HA) polypeptide could sensitise target cells for recognition by class II restricted HA-specific CTL. Treatment of target cells with the lysosomotropic agent chloroquine was shown to abolish recognition of haemagglutinin treated cells by class II MHC restricted HA-specific CTL. Since chloroquine is known to inhibit antigen processing, these results indicate that class II MHC restricted CTL only recognise processed antigen which has bound to class II MHC antigens. That peptide fragments may indeed bind to class II MHC antigens was shown by Babbitt *et al.* (1986) who

demonstrated that a ten amino-acid fragment of hen egg lysozyme bound directly to the murine class II MHC H-2IA<sup>k</sup> antigen. The structure of class II MHC antigens has not yet been studied by X-ray crystallography. However, models based on the amino-acid sequence of class II molecules have indicated that the two amino-terminal domains of the class II  $\alpha$  and  $\beta$  chains (i.e. the  $\alpha 1$  and  $\beta 1$  domains) may form an intermolecular dimer with a structure similar to the  $\alpha 1$  and  $\alpha 2$  region of HLA-A2, thereby creating a binding site for the antigens similar to that in HLA-A2 (Bjorkman *et al.*, 1987a).

Although the minimal requirement for antigen-specific CTL recognition of target cells appears to be the recognition by  $\alpha$  and  $\beta$  chains of the T-cell receptor of antigen in association with the appropriate MHC molecule, it is now clear that a number of other cell-adhesion molecules stabilise the interaction with the target cell. To date, two receptor-ligand pairs of adhesion molecule have been defined. Thus, the CD2 and lymphocyte function associated antigen-1 (LFA-1) receptors (which are expressed on the surface of T-cells) interact with the ligands LFA-3 and intercellular adhesion molecule-1 (ICAM-1) (which are expressed on a variety of cell types), respectively (Dustin *et al.*, 1986; Springer *et al.*, 1987). IFN- $\gamma$  has been shown to increase ICAM-1 expression on a variety of cell types (Dustin *et al.*, 1988). It is possible that the augmentation by IFN- $\gamma$  of ICAM-1 expression on target cells may increase the avidity of CTL-target cell interactions. In man, class I MHC restricted CTL express the cell-surface molecule CD8 (Lyt 2 is the equivalent molecule in the mouse), while class II MHC restricted CTL and T-helper cells express the CD4 molecule (LJT4 in the mouse) (Male *et al.*, 1987). CD8 and CD4 have been shown to interact directly with class I and class II MHC antigens, respectively (Bushkin *et al.*, 1988; Doyle and Strominger, 1987). The interaction of CD4 and CD8



with MHC antigens may also increase the avidity of effector-target cell interactions.

Once a virus specific CTL has undergone recognition of viral antigen on a target cell it is activated to undergo the lytic process. The mechanisms whereby a virus-specific CTL may lyse its target cell are discussed in detail in section 4 of this introduction. Following antigenic stimulation, virus-specific CTL are induced to express receptors for interleukin-2 (IL-2 : T-cell growth factor), and proliferate in response to IL-2 (Kaplan et al., 1984). CTL also release IL-2 and IFN- $\gamma$  on appropriate recognition of antigen on a target cell (Morris et al., 1982; Andrus et al., 1984; Cunningham et al., 1985; Taylor et al., 1985). IL-2 released by virus-specific CTL may bind to receptors for IL-2 on virus-specific CTL and thereby induce proliferation. The fact that virus-specific CTL only express IL-2 receptors and respond to IL-2 on specific recognition of viral antigen may ensure the specificity of the T-cell response. IFN- $\gamma$  released by virus-specific CTL would exert a wide range of immunomodulatory effects, as well as inhibit further replication of virus.

A number of studies have shown that virus-specific CTL play a protective role in vivo. For example, Askonas and coworkers showed that administration of influenza-specific CTL clones to Balb/c mice, immediately after challenge with a lethal dose of the A/X-31 strain of influenza virus, protected mice against lethal infection and reduced the titre of virus present in the lungs (Lin and Askonas, 1981; Taylor and Askonas, 1986).

#### (4) Importance of class II MHC antigens for helper T-lymphocyte function

An essential step in the induction of an immune response to a protein

antigen is the activation of antigen-specific T-helper cells. This activation cannot be induced by free antigen but only by antigen presented by an accessory cell which displays the class II antigens of the MHC. Furthermore, T-helper cells are only activated by accessory cells which possess the same set of class II MHC genes (Schwartz *et al.*, 1985).

The relationship between expression of class II antigens and ability to present antigen has been most studied using the macrophage. These cells do not constitutively express class II MHC antigens, but can be induced to express class II MHC antigens by IFN- $\gamma$  (Unanue, 1981; Beller, 1984). That expression of class II MHC antigen on macrophages correlates with the functional ability of these cells to present antigen was demonstrated by Beller and Ho (1982). In this study it was shown that macrophages isolated from the peritoneal cavity of C57 BL6J mice were both class II MHC antigen positive and able to present Listeria monocytogenes to listeria-specific T-cells. However, when cultured, macrophages no longer expressed class II antigen, and this correlated with an inability to act as antigen presenting cells. The addition of a lymphokine supernatant derived from activated T-cells (and which we now know to contain IFN- $\gamma$ ) to the class II MHC negative cells resulted in the re-acquisition of class II MHC antigen expression and restored the ability to present Listeria antigen to T-cells. In a subsequent study Beller (1984) treated cultured macrophages with various concentrations of the T-cell lymphokine supernatant, in order to study further the relationship between the level of class II MHC antigen induced and ability to present antigen. The results showed that over a concentration range of the lymphokine supernatant, the induction of class II MHC antigen on macrophages was linear, and the ability to present Listeria antigen to T-cells directly correlated with the amount of class II MHC antigen induced. Beller (1984) also showed that recombinant IFN- $\gamma$

induced class II MHC antigen expression on cultured macrophages and restored the ability of the cells to present antigen. Further studies on the induction of class II MHC antigen expression and its role in the antigen presenting ability of the murine P338 D1 macrophage cell line were performed by Zlotnik *et al.* (1983). In these studies the ability of T-cell lymphokines and recombinant IFN- $\gamma$  induced macrophages to present tuberculin-purified protein derivative (PPD) antigen to PPD-specific T-cell hybridomas was assessed by measuring production of IL-2. Both the T-cell lymphokine supernatant and recombinant IFN- $\gamma$  (but not IFN- $\alpha$  or IFN- $\beta$ ) induced class II MHC antigen expression on the cells in a dose-dependant manner, and this was directly related to the ability of the cells to present PPD antigen to T-cells.

That IFN- $\gamma$  can increase class II MHC antigen expression and hence the ability of cells to present antigen to T-cells has also been demonstrated in other systems. Thus, recombinant IFN- $\gamma$  has been shown to increase class II MHC antigen expression on cultured human foreskin capillary endothelial cells, cultured human dermal fibroblasts, and human U-373 MG glioma cells, and also increases the ability of the cells to stimulate the proliferation of allogeneic T-cells (Pober *et al.*, 1983; Takiguchi *et al.*, 1985). IFN- $\gamma$  has also been shown to increase class II MHC antigen expression on human monocytes and also the ability of these cells to stimulate T-cell proliferation in response to tetanus toxoid and to allogeneic monocytes (Becker *et al.*, 1985). That the level of allogeneic and antigen driven proliferation was related to the level of class II MHC antigen expressed at the time of stimulation was demonstrated by incubation of the monocytes with antibody to the class II MHC antigens which inhibited the ability of the cells to stimulate both allogeneic and tetanus toxoid driven proliferation. Interestingly, Becker (1985) also showed that the increased

ability of IFN- $\gamma$  treated monocytes to stimulate T-cell proliferation in response to tetanus toxoid was partly due to a shift in the time course of proliferation. Thus, the response to tetanus toxoid peaked 1 to 2 days earlier when IFN- $\gamma$  treated cells acted as the accessory cells than when untreated monocytes presented the antigen. Treatment of murine vascular endothelial cells with concanavalin-A-conditioned medium (which contains IFN- $\gamma$ ) has also been shown to induce the ability of these cells to present guinea pig basic protein to specifically sensitised lymph node lymphocytes (depleted of macrophages and monocytes). Again the acquisition of antigen presenting ability correlated with an increase in class II MHC antigen expression from otherwise undetectable levels. Furthermore, the importance of class II MHC antigens for antigen presentation was shown by the blocking of proliferation by addition of antiserum to class II MHC antigens (McCarron *et al.*, 1983). IFN- $\gamma$  treatment has also been shown to induce the ability of Schwann cells isolated from rat sciatic nerve (Wekerle *et al.*, 1986a) and murine microglial cells (Frei *et al.*, 1987) to present endogenous myelin autoantigen to myelin-specific T-cells and ovalbumin to ovalbumin-specific T-cells, respectively. Yet again, IFN- $\gamma$  treatment induced the expression of class II MHC antigens and only class II positive cells could present antigen to T-cells. In addition, Todd *et al.* (1985) demonstrated that IFN- $\gamma$  induced class II MHC antigen expression on cultured thyroid epithelial cells, whilst Londei *et al.* (1984) showed that thyroid epithelial cells aberrantly expressing class II MHC antigens were able to present virus peptide antigens to influenza specific T-helper cell clones. This may have implications not only for immune responses to viruses but also for the generation of autoimmune disease.

Matis and coworkers examined the relationship between class II MHC antigen expression on murine splenic cells and the concentration of pigeon

cytochrome c required for the cells to activate cytochrome c specific T-cell clones for proliferation. Through the study of cytochrome c concentration - response curves for T-cells stimulated with splenic cells known to display different amounts of class II MHC antigen, it was shown that the magnitude of the T-cell proliferative response was a function of cytochrome c concentration and the level of class II MHC antigen display (Matia *et al.*, 1982, 1983).

The data discussed above suggest that the acquisition of antigen presenting ability is a consequence of class II MHC antigen expression, and the magnitude of T-cell responses is dependant on both the level of class II MHC antigen displayed and the concentration of foreign antigen present. It is however important to note that there may be other requirements for the induction of a cells antigen presenting ability. This point has been aptly illustrated by Geppart and Lipsky (1987) who investigated the capacity of IFN- $\gamma$  treated HLA-DR expressing human dermal fibroblasts to present streptokinase streptodornase (SKSD) antigen to T-cells. The IFN- $\gamma$  treated fibroblasts were able to take up SKSD antigen, internally process the antigen, and express class II MHC-SKSD antigen complexes on the cell surface but they failed to present antigen to T-cells. Possible reasons for the failure to present antigen were investigated and it was shown that although the class II MHC antigen-SKSD antigen complex was a functionally recognizable element, the fibroblasts were unable to provide an additional accessory signal which was necessary for the antigen induced activation of resting T-cells.

Shimonkevitz *et al.* (1983) studied antigen recognition by murine class II MHC restricted T-cell hybridomas specific for ovalbumin (OVA). These cells were found to recognise OVA presented by untreated antigen presenting B-

lymphoma cells (APBCs) (as assessed by the release of IL-2), but failed to respond when the APBCs were rendered metabolically inert by fixation with glutaraldehyde. When OVA peptides (prepared by digestion with the enzyme trypsin or by cleavage at methionine residues with cyanogen bromide) were used as the stimulating antigen, the T-cell hybridomas could respond whether the APBCs were fixed or not. These results were the first to suggest that class II MHC restricted T-cells require antigen to be fragmented before it can be recognised. The mechanism whereby class II MHC restricted T-helper cells and CTL recognise processed foreign antigen peptide fragments is believed to be identical and has been discussed previously in section c.

T-helper cells constitutively express receptors for interleukin-1 (IL-1) (Lowenthal and MacDonald, 1987). IL-1 is produced by a variety of cell types including macrophages, monocytes, endothelial cells, dendritic cells, and epithelial cells (Male *et al.*, 1987). T-helper cells bind IL-1 on recognition of foreign antigen on an antigen presenting cell and are thus induced to express receptors for IL-2 and to release IL-2, and IFN- $\gamma$  (Male *et al.*, 1987). IL-2 stimulates the proliferation of CTL and T-helper cells which have undergone recognition of antigen (and hence express IL-2 receptors) (Male *et al.*, 1987). The importance of T-helper cells and IL-2 in the generation of virus-specific CTL activity was shown by Brackman *et al.* (1986). In this study, stimulation of human peripheral blood lymphocytes with influenza A virus *in vitro* was found to result in the generation of virus-specific CTL activity. Depletion of T-helper cells from the PBL completely abrogated the generation of virus-specific CTL activity, and activity was restored by the addition of exogenous IL-2. T-helper cells were originally defined in functional assays as being able to induce B lymphocytes to secrete antibody. By using this criterion,

T-helper cells were also shown to interact with B-lymphocytes in a class II MHC-restricted manner (Killar *et al.*, 1987). The binding of T-helper cells to B cells causes the B cells to synthesise receptors for a B-cell growth factor called interleukin-4 (IL-4) (Harlow and Lane, 1988). IL-4 is synthesised by activated T-helper cells (Killar *et al.*, 1987). After binding IL-4, B cells start to proliferate and synthesise cell-surface receptors for a B-cell differentiation factor known as interleukin-5 (IL-5). IL-5 is also produced by activated T-helper cells. The binding of IL-5 to proliferating B cells induces the cells to differentiate into plasma cells (which secrete antibody) and memory cells (Harlow and Lane, 1988). Evidence that class II MHC restricted L3T4<sup>+</sup> T-helper are involved in antibody generation *in vivo* was obtained by Lightman and coworkers. Thus, depletion of L3T4<sup>+</sup> cells from Balb/c spleen cells prior to adoptive transfer to irradiated mice greatly reduced the ability of mice to produce IgG antibody in response to influenza A virus infection (Lightman *et al.*, 1987).

#### (4) Mechanism of cytotoxic T-lymphocyte killing of target cells

With the advent of techniques for cloning T-cell populations, rapid advances have been made in the understanding of the mechanisms of target cell killing by CTL. It is now clear that the killing process can be separated into three distinctly recognisable phases. The first is the interaction between CTL and the target cell, the second is the preparation by the CTL for delivery of its 'lethal hit', and the third is the CTL-dependant destruction of the target cell (Male *et al.*, 1987).

As mentioned above, CTL use the  $\alpha$  and  $\beta$  chains of their T-cell receptor to recognise specific antigen in association with the appropriate class I MHC

antigen on the target cell. Other membrane molecules such as Lys-2/CD8, L3T4/CD4, CD2 and LFA-1 stabilise the interaction with the target cell. The effective adhesion of CTL to target cells is an active process, since it is inhibited by metabolic inhibitors such as azide (Male *et al.*, 1987). It also has a requirement for divalent cations, with  $Mg^{++}$  being more effective than  $Ca^{++}$ . Although effector to target interactions occur in the absence of  $Ca^{++}$ , no lysis occurs because later events are  $Ca^{++}$ -dependent (Male *et al.*, 1987). Once CTL recognition of antigen and adhesion to the target cell has taken place the concentration of intracellular  $Ca^{++}$  in the CTL increases, and the CTL is activated to undergo the lytic process (Ostergaard and Clark, 1987). The CTL begins to reorganise its cytoplasmic constituents such that the Golgi apparatus and cytoplasmic organelles lie between the nucleus and the area of contact with the target cell. The cytoskeletal proteins tubulin and actin also become polarised within the CTL in order to face the target (Male *et al.*, 1987). A broadening of the region of membrane contact with the target cell has been observed and this involves interdigitations of the two membranes (Sanderson and Clauert, 1979). This may provide a greatly increased area of contact with the target cell and thereby improve the efficiency of lysis. Yanelli *et al.* (1986) also reported that CTL formed a uropod which extended the region of interaction. Furthermore, cytoplasmic granules were found to reorientate into the region of contact, and fusions of granules with the cell membrane were observed (Yanelli *et al.*, 1986). Since it had been shown that a marked correlation existed between the presence of cytoplasmic granules and the ability of cloned CTL to lyse target cells (Grossi *et al.*, 1983), and granules isolated from CTL lysed targets in a non-specific  $Ca^{++}$  dependant manner (Podack and Koningsberg, 1984), the observations that granules reorientated and fused with the cell membrane indicated that the secretion of granule contents was involved in the cytolytic mechanism. The major



proteins in CTL granules are a family of serine proteases designated granzymes A to H (Masson and Tschopp, 1987), and a pore-forming protein called perforin (also known as cytolyisin) (Masson and Tschopp, 1985). The fact that proteases were detected in the supernatants of CTL-target conjugates also supports the hypothesis that secretion of granule contents is involved in the cytolytic mechanism (Tschopp and Jongeneel, 1988). Perforin is a hydrophilic single chain protein with a molecular mass of 66 to 75 Kd (Tschopp and Jongeneel, 1988). When secreted from CTL granules, perforin binds to phosphorylcholine on the target-cell membrane (Tschopp *et al.*, 1989), and in the presence of  $Ca^{++}$  polymerises within the cell membrane into an amphiphilic polymeric form (Young and Cohn, 1988). Polymerised perforin (polyperforin) forms tubules in the target cell membrane which have an inner diameter of 16 nanometres (nm), a height of 16 nm, and are composed of 10 to 20 perforin monomers (Tschopp and Jongeneel, 1988; Young and Liu, 1988). These tubules perforate the target membrane and form non-selective transmembrane channels which ultimately lead to cell death (Tschopp and Jongeneel, 1988). Thus, there results an equilibrium of ions across the membrane, and cytoplasmic macromolecules exert an unbalanced osmotic pressure such that water is taken up and the nuclear membrane invaginates. Convolution of the plasma membrane leads to the cell separating into small membrane bound segments called apoptotic bodies. Accompanying the nuclear changes, the chromatin is rapidly degraded into discrete fragments which have multiples of about 200 base pairs of DNA (Male *et al.*, 1987). Pore formation has been shown to be similar in many respects to the channel formation induced by the ninth component of complement (C9) (for review see Tschopp and Jongeneel, 1988), although C9 and perforin clearly differ in their mode of target cell recognition. Thus, C9 insertion is directly dependant on a receptor moiety assembled from the complement components C5b, C6, C7 and C8 on the target cell

membrane, whereas perforin binds to phosphorylcholine on the target cell membrane (Tschopp *et al.*, 1989). After a CTL has damaged its target cell, it can recycle to lyse new targets, hence clearly spares itself from lysis (Young and Liu, 1988). Classic experiments performed some time ago indicate that under certain conditions CTL are effectively lysed by other CTL, implying that CTL must themselves be sensitive to the mechanism they use to kill target cells (Golstein, 1974). However, more recent experiments have suggested that CTL show an unusually high resistance to lysis by effector CTL. Resistance appears to vary in proportion to the lytic potency of the CTL - the more aggressive the CTL, the more resistant it is to other attacking CTL (Kranz and Eisen, 1987; Blakely *et al.*, 1987; Skinner and Marbrook, 1987). Cloned CTL are not only resistant to other effector CTL, but also to lysis by purified perforin, which normally lyses target cells in the presence of  $Ca^{++}$  (Young and Cohn, 1988). It has been speculated that the resistance of CTL to their own perforin may be mediated by a theoretical surface protein known as protectin which rapidly combines with any perforin monomers that get into the cell membrane, and thereby prevents the polymerisation that leads to pore formation (Young and Cohn, 1988).

Although perforin is abundantly present in murine and human CTL lines maintained *in vitro*, detectable levels have not been found in lymphocytes isolated from human blood, in murine peritoneal exudate lymphocytes, or in any tissues so far examined. However, when isolated lymphocytes are stimulated with interleukin-2 *in vitro* they proliferate and begin to synthesise perforin (Young and Cohn, 1988; Young and Liu, 1988). It is possible that perforin may only be present in CTL *in vivo* during disease states which show an increase in interleukin-2 levels.

As mentioned above, CTL granules have been shown to contain a family of proteases designated granzymes A to H. The function of the granzymes is poorly understood. Unlike purified perforin, purified granzymes alone have no cytolytic activity, and there is no evidence to suggest that granzymes act in a proteolytic cascade which leads to the activation of perforin (as is the case in the complement system) (Tschopp and Jongeneel, 1988). The fact that protease inhibitors were found to suppress the lytic activity of CTL does however suggest that proteases may be involved in CTL-mediated killing (Hudig *et al.*, 1984). It has been suggested that granzymes may need to pass through polyperforin tubules in order to have their effect (Male *et al.*, 1987). Furthermore, it has been suggested that granzymes may facilitate the detachment of CTL from the target cell after delivery of the lethal hit has taken place (Tschopp and Jongeneel, 1988).

The ability of some cloned CTL to lyse targets cannot be accounted for solely by the effect of perforin. Thus, Young *et al.* (1987) showed that only four out of eight CTL lines tested contained perforin, whereas all eight were potent killers. Furthermore, subcellular fractions (granules and cytosolic material) of all eight CTL lines were found to be cytotoxic to tumour targets even in the absence of calcium (Young *et al.*, 1987). Trenn *et al.* (1987) also showed that CTL killed target cells even when secretion of granule contents did not take place. These findings clearly indicate that a calcium and perforin independent pathway of killing also exists.

Lymphotoxin was originally described as a cytotoxic activity secreted by activated T-cells (Granger and Williams, 1968). Its role in the cytolytic process has remained controversial. Lymphotoxin has been shown to have a high level of amino-acid sequence homology with the macrophage cytotoxic

factor, tumour necrosis factor (TNF), and to have biological activities indistinguishable from those of TNF, probably because the two proteins share the same receptor (Aggarwal *et al.*, 1985). Therefore, they are now called TNF- $\alpha$  (formerly TNF) and TNF- $\beta$  (formerly lymphotoxin). The proposed role of TNF- $\beta$  in CTL-mediated cytotoxicity is based on the fact that many CTL lines secrete TNF- $\beta$  when incubated with target cells, and that TNF- $\beta$ -containing cell supernatants induce DNA degradation in some tumour cell lines (Tschopp and Jongeneel, 1988). However, several lines of evidence argue against a direct role for TNF- $\beta$  in target cell killing by CTL. First, it is known that many cell lines that can be killed by CTL are refractory to the toxic effects of TNF- $\beta$ . Second, the appearance of TNF mRNA after antigenic stimulation and secretion of the protein takes several hours, and CTL kill their targets within minutes of contact. Third, several CTL lines have been found to kill their targets without producing detectable quantities of TNF- $\beta$  mRNA or protein (Tschopp and Jongeneel, 1988).

As mentioned previously, adhesion of CTL to its target cell ultimately results in a degradation of the target cell genome into DNA fragments which consist of multiples of 200 base pairs. The observations made by Wyllie (1980) that treatment of cortisone-sensitive thymocytes with glucocorticoid hormone resulted in a similar pattern of DNA fragmentation led Ucker (1987) to postulate that the CTL and glucocorticoid mediated cytolytic pathways may share a common mechanism. Ucker reasoned that if the CTL and glucocorticoid mediated cytolytic pathways do indeed share a common mechanism, then genetic defects which abolish cytotoxicity by glucocorticoids must also confer resistance to killing by CTL. The susceptibilities of a glucocorticoid-sensitive thymoma cell line (549), and a glucocorticoid-resistant mutant derived from this cell line, to lysis by alloreactive CTL

were thereby examined. The results obtained clearly showed that whilst both cell-types were effectively recognised by the CTL (as determined by conjugate formation), only the glucocorticoid sensitive cells were killed by the CTL. A single step spontaneous reversion event was also found to restore sensitivity of the glucocorticoid-resistant cells to both glucocorticoid and CTL lysis. These observations led Ucker (1987) to conclude that a single genetic lesion was responsible for the resistant phenotype, and that the normal gene activity of this locus in a target cell must be required for the target cell to respond (by dying) to the cytolytic signal provided by CTL or glucocorticoids. Hence, target cells play an active role in their own death, and CTL act by inducing the autolytic process.

In summary, it is clear that killing by CTL is complex, and probably involves multiple mechanisms as well as multiple mediators. It is possible that these may act either independently or synergistically in the cytolytic process.

#### (5) Role of IFNs in vivo

It is now widely accepted that IFNs constitute an integral part in host defence against viral infection. However, much of the evidence has been indirect; based on temporal associations between the presence of virus and IFN in different tissues, and the fact that administration of exogenous IFN can confer a marked protection in virus-infected animals and man (for review see Finter and Oldham, 1985). While such studies indicate the possible importance of IFNs in vivo they do not allow firm conclusions to be made as to the actual role of IFNs produced endogenously during the course of infection. More direct evidence for the role of endogenous IFN

in resistance to disease has been obtained in comparatively few studies by monitoring the effect of neutralising anti-IFN- $\alpha\beta$  antibody on the course of disease. Fauconnier (1970, 1971) was the first to show that intravenous administration of anti-IFN- $\alpha\beta$  serum resulted in an earlier and increased incidence of mortality in mice infected intramuscularly with small amounts of Semliki Forest virus (SFV). Thus, mice infected with 0.2 LD<sub>50</sub> of SFV survived, whereas over 50% of infected mice died when treated with anti-IFN- $\alpha\beta$  serum. Titres of virus in the brains of SFV-infected mice treated with anti-IFN- $\alpha\beta$  serum were also found to be about ten times higher than in control mice. Fauconnier concluded that the anti-IFN- $\alpha\beta$  serum neutralised IFN- $\alpha\beta$  produced in response to SFV-infection and thus permitted extensive viral multiplication to take place. Subsequently, administration of anti-IFN- $\alpha\beta$  serum was also shown to exacerbate disease in mice infected with encephalomyocarditis virus (Gresser *et al.*, 1976a; Guillon and Gresser, 1978), polyoma virus (Gresser *et al.*, 1979), mouse hepatitis virus type 3 (Virelizier and Gresser, 1978), rabies virus (Marcovitz *et al.*, 1986), Moloney sarcoma virus, vesicular stomatitis virus, and Newcastle disease virus (Gresser *et al.*, 1976b). These observations indicate that IFN- $\alpha\beta$  has an important role in the response of the mouse to a number of viruses which exhibit different pathogenesises. Gresser *et al.* (1976b) showed that intranasal and intravenous administration of anti-IFN- $\alpha\beta$  serum had no effect on the course of influenza (strain PR8) virus infection of mice. Although these observations suggest that IFN- $\alpha\beta$  has no role in the immune response to influenza virus infection, it is possible that the absence of effect may have been related to an inability of the globulin to diffuse to the tracheobronchial epithelium and thus to neutralise IFN- $\alpha\beta$  at the site of viral replication (Gresser *et al.*, 1976b). Berlardelli *et al.* (1984) obtained evidence that small amounts of IFN- $\alpha\beta$  may be present in healthy mice. Thus, vesicular stomatitis virus and encephalomyocarditis virus were

found to multiply in only a small percentage of peritoneal macrophages freshly explanted from healthy adult mice. However, when mice were intraperitoneally treated with anti-IFN- $\alpha\beta$  serum several days prior to harvesting peritoneal macrophages the viruses multiplied to high titres and most cells were infected. These results indicate that there may be low levels of endogenous IFN- $\alpha\beta$  that contribute to host defense by maintaining some cells in an antiviral state. Bocci (1988) speculated that these low levels of IFN- $\alpha\beta$  in healthy mice may be stimulated, at least in part, by lipopolysaccharide derived from bacteria which colonise the gut. IFN- $\alpha\beta$  cannot however normally be detected in peritoneal washings, splenic extracts or serum from healthy mice (Grasser *et al.*, 1983). IFN- $\alpha\beta$  also appears to play a role in the immune response against tumours since administration of anti-IFN- $\alpha\beta$  serum has been shown to enhance the transplantability of a number of different murine tumours, as determined by an increase in the number of tumour bearing mice and a decrease in survival time (Grasser *et al.*, 1983).

Although IFN- $\alpha\beta$  has a beneficial role in the immune response of the mouse to a number of virus-infections, there are also instances in which IFN- $\alpha\beta$  does not protect the mouse but is actually responsible for the lethal outcome of virus-infection. Thus, Riviere *et al.* (1977) showed that although administration of anti-IFN- $\alpha\beta$  serum enhanced lymphocytic choriomeningitis virus (LCMV) multiplication 100-fold, it neutralised circulating IFN- $\alpha\beta$  and inhibited the decreased growth, liver necrosis and death normally observed in LCMV-infected newborn mice. Similarly, Pfau *et al.* (1983) showed that administration of anti-IFN- $\alpha\beta$  serum enhanced titres of the 'aggressive' strain of LCMV in adult mice but the mice survived the lethal outcome observed in control mice.

Until very recently there were no preparations of antibody available which specifically neutralised IFN- $\gamma$ . Consequently, only a small number of studies have used anti-IFN- $\gamma$  antibody to examine the role of IFN- $\gamma$  produced endogenously during the course of disease in mice. Buchmeier and Schreiber (1985) were the first to show that endogenous IFN- $\gamma$  has a beneficial role in the immune responses of the mouse. Thus, administration of anti-IFN- $\gamma$  monoclonal antibodies to mice infected intraperitoneally with sublethal doses of *Listeria monocytogenes* was shown to completely inhibit the generation of activated macrophages in the peritoneal cavity, abrogate the clearance of bacteria from the spleen and peritoneal cavity, and lead to the death of at least some mice. Subsequently, administration of anti-IFN- $\gamma$  monoclonal antibodies was also shown to abrogate both protective immunity against malaria sporozoite challenge (Schofield *et al.*, 1987) and tumour immunity against Rous sarcoma virus-induced murine sarcomas (Prat *et al.*, 1987). The use of anti-IFN- $\gamma$  monoclonal antibodies has revealed a role for IFN- $\gamma$  in both inflammatory responses and the pathogenesis of autoimmune disease. Thus, administration of anti-IFN- $\gamma$  monoclonal antibodies has been shown to alleviate systemic lupus erythematosus disease (Jacob *et al.*, 1987), and inhibit the general Shwartzman reaction (endotoxic shock syndrome) in mice treated with two separate injections of lipopolysaccharide (Billiau *et al.*, 1988). Skoglund *et al.* (1988) studied the effects of anti-IFN- $\gamma$  monoclonal antibodies on the delayed type of hypersensitivity (DTH) reaction induced by administration of 2,4-dinitro-1-fluorobenzene onto the ears of sensitised Lewis rats. Anti-IFN- $\gamma$  monoclonal antibodies were found to inhibit the induction of class II MHC antigen expression on keratinocytes during the DTH reaction but in contrast increased the swelling of ears. These observations suggest that endogenous IFN- $\gamma$  induced class II MHC antigen expression on keratinocytes during the DTH reaction, and that IFN- $\gamma$  had a suppressive role in the local immune



response. Jephthah-Ochola et al. (1988) showed that anti-IFN- $\gamma$  monoclonal antibodies inhibited the ability of lipopolysaccharide to induce class I and class II MHC antigen expression in the kidney of mice. These observations indicate that lipopolysaccharide may stimulate endogenous IFN- $\gamma$  production which induces MHC antigen expression in the kidney. To date, there have been no reports in which anti-IFN- $\gamma$  antibodies have been used to study the role of IFN- $\gamma$  produced endogenously during viral disease. Hopefully this situation will soon be remedied now that anti-IFN- $\gamma$  antibodies are more widely available.

#### (4) Semliki Forest virus

##### (a) History, classification, structure and replication

Semliki Forest virus (SFV) was originally isolated from a pool of 130 female mosquitoes caught in Swamba, Uganda in 1942 (Smithburn and Haddock, 1944). Semliki means 'I do not know' - the reply given by natives when asked the name of the forest (Rowson et al., 1981).

SFV is a species in the genus alphavirus of the family Togaviridae (Porterfield et al., 1978). It is also described as an arbovirus (arthropod borne virus) since it is transmitted by a mosquito vector and infects vertebrates (Rowson et al., 1981). The natural vector-host cycle of the virus is not known although antibodies have been found in man and wild primates in Uganda, Mozambique, Cameroon, Central African republic, Kenya, Nigeria, Borneo and Malaya (Rowson et al., 1981). SFV is not associated with any disease, and is generally considered to be non-pathogenic for man although a fatal case of encephalitis in a laboratory worker with purulent bronchitis has been associated with the virus (Willems

et al., 1979).

SFV is an enveloped virus with a diameter of 50-60 nm. The virus consists of an icosahedral nucleocapsid surrounded by a lipid bilayer (Murphy, 1980). The nucleocapsid is a complex of capsid (C) proteins and the genome (Garoff et al., 1980). SFV contains as its genome a single strand of RNA of positive polarity and is thus classified as a group IV virus using the Baltimore classification of viruses (Sawicki and Sawicki, 1980; Primrose and Dimmock, 1980). The SFV genome has a sedimentation coefficient of 42S, and has been mostly sequenced (Levin and Friedman, 1971; Simons et al., 1982). The lipid bilayer has external glycoprotein spikes each consisting of three different polypeptides : E1, E2 and E3, which exist as a trimer (Garoff et al., 1974; Ziemiecki and Garoff, 1978). E2 spans the lipid bilayer such that the hydrophobic carboxyterminus interacts with the nucleocapsid (Garoff and Simons, 1974; Ziemiecki and Garoff, 1978). E1 is anchored to the lipid bilayer by its hydrophobic carboxyterminus (Ziemiecki and Garoff, 1978).

SFV attaches to cells by its glycoprotein spikes (Osterrieth and Calberg-Bacq, 1966). Helenius et al. (1978) provided evidence that SFV binds to class I MHC antigens on the surface of human and murine cells. However, Oldstone et al. (1980) showed that SFV replicated equally well in murine cells that both expressed, and did not express, class I MHC antigens, indicating that class I MHC antigens may not be the only cell-surface receptor for SFV. SFV is thought to enter cells by receptor mediated endocytosis. Thus, Helenius et al. (1980) showed that SFV was incorporated into coated pits on the surface of baby hamster kidney-21 (BHK-21) cells, which invaginated to form coated vesicles inside the cell. Vesicles bearing endocytosed virions subsequently fused with lysosomes. The lipid

bilayer of the virus was found to fuse with lysosomal membranes when the pH was 6 or lower, and it was proposed that this fusion may result in the release of the nucleocapsid in the cytoplasm. Replication of SFV takes place in the cytoplasm (Grimley et al., 1968). The nucleocapsid is uncoated and the released genomic RNA translated by host cell ribosomes to produce a precursor polypeptide which is subsequently cleaved to yield non-structural polypeptides which form a viral coded RNA polymerase (Kariainen and Soderlund, 1978). The virus polymerase then synthesizes complementary strands of the genome (42S negative sense RNA) which are used as a template for the synthesis of two species of positive sense RNA : new 42S RNA genomes and a subgenomic 26S RNA which codes for the structural E1, E2, E3 and C polypeptides of the virus (Sawicki and Sawicki, 1980). The newly synthesized capsid protein is located in the cell cytosol, where it binds to 42S RNA to form new nucleocapsids. In contrast, the envelope proteins are inserted into the membrane of the endoplasmic reticulum, where they become glycosylated (Alberts et al., 1983). Remaining membrane-bound at all times, the envelope proteins are transported first to the Golgi apparatus (where they are further glycosylated) and then to the plasma membrane. As a result of specific interaction with the envelope proteins, the nucleocapsid becomes wrapped in a portion of the plasma membrane that is highly enriched with virus envelope proteins, and subsequently buds out of the cell in such a way as to acquire a complete envelope (Alberts et al., 1983).

#### (b) SFV infection of the laboratory mouse

SFV was discovered by the ability of mosquito extracts to cause a lethal encephalitis in mice when inoculated intracerebrally. Initially, mice died at around day 6 post-infection, but after 40 passages mice inoculated with

a high dose of virus died at around day 2 post-infection (Smithburn and Haddow, 1944; Atkins *et al.*, 1985). Seamer *et al.* (1967) studied the course of SFV infection in Porton mice infected with a thirteenth mouse brain passage of the original isolate. Following footpad inoculation high titres of virus were found in the blood, spleen, brain, liver and popliteal lymph nodes, but only the brain and spinal cord showed histological lesions. Titres of virus were higher in the spleen and brain than in blood-observations which led Seamer and coworkers to conclude that the brain and spleen were sites of virus multiplication. Although the spleen was a site of virus multiplication its importance in this respect was overshadowed by the brain where considerably higher titres of virus were found later in the course of infection. SFV is thus described as being neurotropic.

Bradish *et al.* (1971) studied the virulence of Smithburn and Haddow's original strain and three other isolates (isolated by other workers from pools of mosquitos in Nigeria and Mozambique, and from human blood in Surinam) which had been passaged in mice, and in some cases also in chick embryo fibroblasts *in vitro*, to different extents. Although these strains could not be distinguished serologically, they were found to differ markedly in their virulence for mice, guinea-pigs and rabbits when administered by the intraperitoneal, intracerebral or respiratory routes. For Porton mice, neonatal animals were susceptible to all strains, but adult mice infected intraperitoneally were susceptible only to virulent strains. There was also found to be a sharp transition to resistance to the avirulent A7 strain at 16 to 17 days of age. Infection with avirulent strains then led to benign replication of the virus and protection of the host against challenge with a virulent strain (Bradish *et al.*, 1972; reviewed by Atkins *et al.*, 1985). Several studies have utilised strains of

defined virulence in an attempt to relate virulence to properties of the virus. Puustai *et al.* (1971) compared the multiplication of a virulent strain (V13) to that of an avirulent strain (A7) in weanling mice. They showed that, following intraperitoneal infection, the two strains multiplied similarly in the early stages of infection. A viraemia of similar titre occurred, which was followed by invasion of the brain. However, V13 appeared in the brain 12 hours before A7 and increased in titre until the death of the mice, whereas A7 reached a peak in the brain and then declined. Subsequent studies also showed that the initial patterns of infection in brain and blood following avirulent or virulent infection were indistinguishable (Bradish and Allner, 1972; reviewed by Atkins *et al.*, 1983). Pathak *et al.* (1976) carried out electron-microscopic studies of the brains of SFV-infected mice. Development of SFV in the brain was found to be similar in weanling and adult mice infected with the virulent L10 strain, and in weanling mice infected with the avirulent A7(74) strain. Mature virus could not however be seen in the brains of adult mice infected with the avirulent A7(74) strain. For the case of weanling mice infected with the virulent L10 strain, replicating virus was found to be associated predominantly with neurons. Subsequently, replicating virus was also found in oligodendrocytes within the brains of both adult mice infected with the virulent L10 strain (Pathak and Webb, 1983) and weanling mice infected with the avirulent M9 mutant of SFV (generated by mutation of the virulent L10 strain with N-nitro-N-methyl-N'-nitroseguanidine : Barrett *et al.*, 1980) (Sheahan *et al.*, 1983). Replicating virus was not found associated with neurons in weanling mice infected with the avirulent A7 strain or M9 mutant (Sheahan *et al.*, 1983; Gates *et al.*, 1985). Atkins and Sheahan (1982) studied the cytopathogenicity of the virulent L10 strain and four avirulent mutant strains (M4, M9, M103, M136: Barrett *et al.*, 1980) for G26-24

oligodendroglioma cells and C1300 neuroblastoma cells in vitro. They showed that the virulent L10 strain and four avirulent mutants produced a rapid cytopathic effect in G26-24 cells. In contrast, C1300 cells infected with the avirulent mutants were found to survive infection, and only the virulent L10 strain produced a cytopathic effect. Subsequent studies also showed that the cytopathogenicity of the avirulent A7 strain was reduced compared to the virulent L10 strain in C1300 cells, but not in G26-24 cells (Atkins, 1983). Similarly, Gates et al., (1985) showed that the virulent L10 strain multiplied better in cultures of neurons, prepared from newborn mice, than did the avirulent A7 strain or H9 mutant. Furthermore, Bruce et al. (1984) showed that mixed primary brain cell cultures, derived from newborn rats, became depleted of oligodendrocytes following infection with the avirulent A7 strain. Administration of myocrisin (sodium aurothiomalate) to adult mice has been shown to potentiate infection by the avirulent A7 strain, and to thus convert a normally avirulent infection into a virulent one (as determined by increased mortality, mean survival and neuronal destruction) (Allner et al., 1974; Bradish et al., 1975b). Myocrisin treatment did not depress the antibody response and it was suggested that macrophage phagocytic activity may have been inhibited (Allner et al., 1974; Bradish et al., 1975b). Myocrisin has been shown to stimulate membrane proliferation on brain cells and to inhibit lymphocyte proliferation. However, it is not yet clear which effects of myocrisin were actually involved in the change of pathogenesis (for review see Atkins et al., 1985). In summary, it does appear that the lethal event in virulent SFV-infection is the destruction of neurons. Limited damage to oligodendrocytes during avirulent SFV-infection is probably tolerated by the mouse and arrested by immune intervention before a lethal threshold of damage can be exceeded.

Infection of adult mice with avirulent SFV results in the formation of demyelinating lesions in the brain (Jagelman *et al.*, 1978; Suckling *et al.*, 1978; Chew-Lim *et al.*, 1978; Webb *et al.*, 1979; Sheahan *et al.*, 1981; Kelly *et al.*, 1982; Pathak *et al.*, 1983). The pathogenesis of demyelination in SFV infection has been subject to a number of studies and some of the data presented is contradictory. Chew-Lim *et al.* (1977) reported that irradiation of mice, prior to infection with the A7(74) strain of SFV, resulted in an increased severity of demyelination and increased titres of virus in the brain. Similar observations were made when the same strain of virus was used in athymic (T-cell deficient) nude mice (Chew-Lim, 1979). These workers concluded that demyelination in A7(74) SFV-infection resulted from direct viral activity rather than from an autoimmune reaction. In contrast, Jagelman *et al.* (1978) and Fazakerley *et al.* (1983, 1987) found no evidence of demyelination in nude mice infected with the A7(74) strain of SFV, while demyelination was observed in similarly infected heterozygous immune-competent litter mates. It was concluded from these studies that a T-cell mediated immunological response was involved in the development of demyelination in immunocompetent mice. Berger (1980) reported that although demyelination occurred in nude mice infected with the A7 strain of SFV, it was reduced in severity as compared to immune-competent mice. Demyelination induced by the A7(74) strain and M9 and M136 mutants of SFV has been shown to be accompanied by an infiltration of leukocytes into the brain (Sheahan *et al.*, 1981, 1983; Kelly *et al.*, 1982; Pathak *et al.*, 1983). Reconstitution of T-cells and/or B cells to immunosuppressed mice, and of T-cells to nude mice, prior to infection with the A7 and A7(74) strains of SFV has also been shown to induce the formation of demyelinating lesions (Berger, 1980; Fazakerley *et al.*, 1983, 1987). These observations indicate that T and B cells play a role in demyelination.

The role of the immune system in recovery from SFV infection has also been subject to a number of studies. It is now clear that infection of mice with SFV results in the endogenous production of IFN- $\alpha\beta$ . Bradish and co-workers found that in Porton mice infected intraperitoneally with the virulent V10, virulent V12, avirulent A7, and avirulent A7(74) strains of SFV, the amount of IFN- $\alpha\beta$  in serum rose from an undetectable level, peaked at around 36 hours post-infection, and then declined back to an undetectable level by day 5 post-infection (Bradish and Allner, 1972; Bradish *et al.*, 1975a). Similarly, Fleming (1977) detected IFN- $\alpha\beta$  in the serum of Porton mice infected intraperitoneally with the virulent L10, virulent V14 and avirulent A7(74) strains of SFV, and found that maximal levels were present at around 24 hours post-infection. In this study Fleming also found that at 24 hours post-infection the level of IFN- $\alpha\beta$  in the spleen was nine times higher than in serum, an observation which led to the conclusion that the spleen may be a site of IFN- $\alpha\beta$  secretion. Bradish and Allner (1972) and Fleming (1977) monitored the amount of IFN- $\alpha\beta$  in the brains of Porton mice infected intraperitoneally with the virulent L10, virulent V14, avirulent A7 and avirulent A7(74) strains of SFV, and found that little IFN- $\alpha\beta$  was present early in infection. At 24 hours post-infection with the avirulent A7(74) strain of SFV, the levels of IFN- $\alpha\beta$  in the brain were only one twentieth of those in serum. Gaten *et al.* (1976) found large amounts of IFN- $\alpha\beta$  in the brains of Swiss A<sub>2</sub>G mice 24 hours after intracerebral infection with the avirulent A7(74) strain of SFV, and showed that the amount of IFN- $\alpha\beta$  present declined to an undetectable level after day 3 post-infection. As mentioned previously, IFN- $\alpha\beta$  produced endogenously during SFV-infection of mice appears to have a protective role since intravenous administration of anti-IFN- $\alpha\beta$  serum to Swiss mice immediately following intramuscular infection with low doses of SFV (strain not specified) resulted in earlier and higher mortality (Faucomnier, 1970,



1971). Finter (1966) demonstrated that intramuscular administration of exogenous natural IFN- $\alpha\beta$  protected Alderley Park strain I mice against lethal doses of the MS strain of SFV injected into the peritoneum. Intraperitoneal administration of exogenous natural IFN- $\alpha\beta$  and double-stranded RNA (an inducer of IFN- $\alpha\beta$ ) to Swiss A<sub>2</sub>C mice and Balb/c mice has also been shown to reduce the efficiency of intraperitoneal infection with the virulent L10 and avirulent A7(74) strains of SFV (Bradish and Titmuss, 1981). To date, no studies have monitored levels of IFN- $\gamma$  present in SFV-infected mice, or have administered anti-IFN- $\gamma$  antibody to determine the role of endogenously produced IFN- $\gamma$ . The generation of SFV-specific T-cells and antibody in SFV-infected mice, and the possible importance in recovery are discussed in detail in chapter 8.

#### (7) Aims of project and introduction to the experimental system

It is now well established that CTL and T-helper cells only recognise foreign antigens presented in association with antigens of the MHC. Cells within the brain differ from most other cells in the body in that they do not usually express detectable amounts of MHC antigen. However, like other organs of the body the brain can suffer from infection with viruses and other microorganisms and thus requires protection by the immune system. It is not yet known whether virus-infected brain cells are able to participate in T-cell mediated immune reactions. IFNs have been shown to increase MHC antigen expression on a variety of cell types. Several studies have also demonstrated that IFNs increase the ability of cells to participate in antigen specific T-cell mediated immune reactions, and that this increased ability correlates with an augmentation of MHC antigen display on the cells. IFNs- $\alpha$  and - $\beta$  are released by most cell types when infected with virus, and IFN- $\gamma$  is released by T-cells on specific recognition of viral

antigen on cells. It is possible that IFNs- $\alpha$ , - $\beta$ , and - $\gamma$  within a virus infected brain may modulate the ability of brain cells to participate in virus-specific T-cell mediated immune reactions.

The major aims of this thesis are:

- (i) To investigate whether virus-infected brain cells participate in class I and class II MHC restricted virus-specific T-cell mediated immune reactions in vitro.
- (ii) To investigate the effect of IFNs on class I and class II MHC antigen expression by brain cells in vitro.
- (iii) To investigate the ways in which IFNs may modulate the susceptibility of brain cells to infection by virus in vitro.
- (iv) To investigate the ways in which IFNs may modulate the ability of virus-infected brain cells to participate in virus-specific T-cell mediated immune reactions in vitro.

SFV was chosen for use in these studies since it is neurotropic and the pathogenesis of disease in mice is reasonably well understood. Studies on the interactions between SFV-infected brain cells and SFV-specific T-cells may also provide an insight into the mechanism by which SFV-infection triggers an apparently T-cell mediated autoimmune reaction which results in demyelination. This may assist in the understanding of multiple sclerosis in man, which is speculated to have a viral aetiology.

The brain cells chosen for use in these studies were primary cultures of astrocytes, G26-24 oligodendrogloma cells, and C1300 neuroblastoma cells. Astrocytes were chosen since nearly pure primary cultures of these cells can be prepared from the brains of newborn mice with little difficulty - a

factor which facilitates routine use (McCarthy and Vellis, 1980; Fontana *et al.*, 1982; Gates *et al.*, 1985). In contrast, cultures of oligodendrocytes and neurons are much more difficult to prepare as pure and stable populations, and require laborious techniques (McCarthy and Vellis, 1980; Suzumura *et al.*, 1984; Snyder *et al.*, 1980; Gates *et al.*, 1985). This makes the routine use of oligodendrocytes and neurons more difficult. The G26-24 oligodendrogloma and C1300 neuroblastoma tumour cell lines were thereby chosen as representative cell types. The G26-24 oligodendrogloma cell line was cloned in 1975 by Sundarraj and coworkers (Sundarraj *et al.*, 1975) from a glial cell tumour (G26) of central nervous system origin induced by methylcholanthrene treatment of the C57 BL/6 mouse (Zimmerman, 1955). G26-24 cells are referred to as oligodendrogloma cells since they exhibit some (but not all) of the properties associated with immature oligodendroglial cells (Dawson, 1979). The C1300 neuroblastoma cell line was cloned from a spontaneous tumour which appeared around the spinal cord of an A/J mouse (Schubert *et al.*, 1969). C1300 cells have been shown to possess some properties of a mature neuron (Schubert *et al.*, 1969). It is important to note that whilst G26-24 and C1300 cells provide a convenient model system, they clearly will not possess characteristics identical to normal oligodendrocytes and neurons.

Neurons receive, conduct, and transmit signals within the nervous system. In the brain, astrocytes and oligodendrocytes support the activity of neurons. Astrocytes provide both mechanical and metabolic support for the complex neuronal circuits; they synthesise and degrade neuronally important compounds and help to control the ionic composition of fluids surrounding neurons. Oligodendrocytes form insulating myelin sheaths around neurons and thereby increase the speed and efficiency of signal transmission (Alberts *et al.*, 1983).

The experimental aims mentioned above will yield information about the ways in which SFV-infected brain cells may participate in SFV-specific T-cell mediated immune reactions in vitro. However, these studies may have no relevance to the situation in vivo during SFV-infection of mice. Studies will therefore be extended to the in vivo system to determine whether IFN- $\alpha\beta$  and IFN- $\gamma$  are actually present within the brains of SFV-infected mice. Class I and class II MHC antigen expression by cells within the brains of SFV-infected mice will also be monitored. Furthermore, to determine whether IFN- $\gamma$  produced endogenously during SFV-infection of mice has any role, R4-6A2 rat-anti-murine IFN- $\gamma$  neutralising monoclonal antibody will be administered to mice, prior to infection, and the effect on the clinical course of SFV disease studied. The R4-6A2 monoclonal antibody was chosen since it was the only neutralising monoclonal antibody to IFN- $\gamma$  available (Spitalny and Havall, 1984), and a polyclonal antiserum to recombinant IFN- $\gamma$  (recombinant to exclude the possibility of contamination with other lymphokines which may be present in preparations of natural IFN- $\gamma$ ) was not available.

## Chapter 2

### Materials and Methods

#### Materials

##### (A) Medium

Dulbecco's modification of Eagles minimum essential medium (DMEM), the Glasgow modification of Eagles minimum essential medium (GMEM), Roswell Park Memorial Institute (RPMI) 1640, and 2 x 199 were all obtained from Gibco Ltd and prepared by Mrs B Wood (University of Warwick). Foetal calf serum (FCS) was obtained from both Gibco Ltd and Northumbria Biologicals Ltd and was heat-inactivated for 45 minutes at 56°C in order to inactivate complement, prior to use. Newborn calf serum (NBS) was obtained from Gibco Ltd. All media were buffered with bicarbonate to pH 7.0, and were supplemented with glutamine (2 mM) (Flow Laboratories Ltd) Penicillin (60 µg/ml) and streptomycin (100 µg/ml) from Glaxo Laboratories Ltd. GMEM was also supplemented with non-essential amino-acids (NEAA) obtained from Sigma. For GMEM BHK, GMEM was supplemented with tryptose phosphate broth (2.9 mg/ml) obtained from Difco Ltd. For RPMI 1640 complete, β-mercaptoethanol (BDH Chemicals Ltd) was added to RPMI 1640 medium to a final concentration of 10<sup>-5</sup> M.

##### (B) Cell lines

All cell lines used were stored long term in liquid nitrogen (see methods). When required cells were thawed and maintained in continuous culture for at least 14 days before use.

1. Baby hamster kidney cells (BHK-21) were obtained from Flow Laboratories Ltd. BHK-21 cells were routinely grown in either 75 cm<sup>2</sup> or 150 cm<sup>2</sup> tissue culture flasks (obtained from Costar Ltd) containing 15 ml and 40 ml respectively of DMEM BHK supplemented with 10% FCS. Cells were subcultured when a confluent monolayer had formed. For subculture of cells in 150 cm<sup>2</sup> flasks, medium was aspirated and the monolayer washed with 10 ml warm PBS (see materials section I) containing 0.02% diaminoethanetetra-acetic-acid (EDTA, obtained from Fisons plc) followed by 5 minutes incubation with 10 ml of the same solution containing 1 mg/ml trypsin (obtained from Flow Laboratories Ltd). Cells were harvested by pipetting into 10 ml medium, pelleted by centrifugation at approximately 3,000 rpm for 5 minutes in an MSE minor bench centrifuge, and resuspended in 10 ml fresh medium. Fresh tissue culture flasks were then seeded with 1 ml of cell suspension (hence, subcultured 1:10). Cells were incubated at 37°C in a Wedge incubator with an humidified atmosphere of 5% carbon dioxide in air. Cells growing in 75 cm<sup>2</sup> flasks were subcultured in an identical manner, with the exception that the volumes of EDTA/PBS and EDTA/PBS/trypsin solutions were halved.
2. Murine neuroblastoma cells (C1300) derived from the A/J mouse (Augusti-Tocco and Sato, 1969) were brought to this department by Dr D J Maudsley (University of Warwick). C1300 cells were routinely grown in DMEM supplemented with 10% FCS. Incubation and subculture conditions as for BHK-21 cells.

3. Murine oligodendrogloma cells (G26-24) derived from the C57 BL/6 mouse (Sundarraj ~~et~~ al., 1973) were brought to this department by Dr D J Maudsley (University of Warwick). G26-24 cells were grown in DMEM supplemented with 10% FCS. Incubation conditions and subculture (1:6) as for BHK-21 cells.
4. Murine 1929 fibroblasts derived from the C3H mouse were brought to this department by Prof D C Burke (currently at University of East Anglia). 1929 cells were grown in GMEM NEAA supplemented with either 10% FCS or NBES. Incubation and subculture as for BHK-21 cells, into 1 litre glass bottles (obtained from Flow Laboratories).
5. Murine ELA lymphoblastoid cells derived from the C57 BL/6 mouse, were obtained from the American Tissue Culture Collection (ATCC), clone TIB 39. ELA cells were grown in 25 cm<sup>2</sup> tissue culture flasks (obtained from Costar Ltd) containing 10 ml RPMI 1640 supplemented with 10% FCS. Cells grew in suspension and were subcultured at a 1:10 dilution. Incubation conditions as for BHK-21 cells.
6. Murine mastocytoma cells (P815) derived from the DBA/2 mouse, were obtained from the ATCC, clone TIB64. Media, incubation conditions and subculture as for ELA cells.
7. Murine EDM4 lymphoblastoid cells were obtained from Mr M J Blackman (currently at National Institute for Medical Research, London). Media, incubation conditions and subculture as for ELA cells.
8. Murine myelomonocyte cells (WEHI 3b) derived from the Balb/c mouse, were brought to this department by Dr D J Maudsley and were originally

obtained from Dr F Kaye (London School Hygiene and Tropical Medicine, London). Media, incubation conditions and subculture as for EL4 cells.

9. Murine Yac-1 lymphoblastoid cells derived from the A/Sn mouse, were obtained from Mr M J Blackman (currently at National Institute for Medical Research, London). Media, incubation conditions and subculture as for EL4 cells.

#### (C) Virus

The virulent L10 strain of Semliki Forest Virus (SFV) (Bradish *et al.*, 1971) was originally obtained from Dr R Fitzgeorge and Dr C Bradish (Porton Down, Wiltshire, UK). Stocks used in the experiments presented in this thesis were prepared by myself from a seed stock of mouse brain SFV as described in methods section A. The avirulent A7(74) strain of SFV (Bradish *et al.*, 1971) was obtained from Dr A Barrett (currently at the Department of Biological Sciences, University of Surrey) and all stocks used were prepared by Mr M J Blackman (currently at National Institute for Medical Research, London).

#### (D) Mice

Male and female 5 to 6 week-old A/J (H2<sup>a</sup>) and C57 BL/6 (H2<sup>b</sup>) mice were obtained from Bantin and Kingman. Male and female 5 to 6 week-old Balb/c (H2<sup>d</sup>), DBA/2 (H2<sup>d</sup>), B10.A.(5R) (H2<sup>i5</sup>) and MFI nu<sup>+</sup>/nu<sup>+</sup> nude mice were all obtained from Olac Ltd. Male and female C3H/He (H2<sup>k</sup>) mice were obtained from a breeding colony present within this department, and which was originally established using breeding pairs supplied by Bantin and Kingman.



#### (E) Interferons

Murine natural IFN- $\alpha\beta$ , specific activity  $4.0 \times 10^6$  units/mg protein was purchased from Lee Biomolecular. Murine recombinant IFN  $\beta$  and  $\gamma$  were prepared in this laboratory from chinese hamster ovary cells transfected with the mammalian expression vector pKSV<sub>10</sub> containing a cDNA copy of the IFN- $\beta$ , or  $\gamma$  mRNA (Morris and Ward, 1987). The IFN present in supernatants harvested from the transfected cells was then partially purified by affinity chromatography on cibricron blue sepharose (obtained from Sigma) by Mr F D Shaw (University of Warwick). The resultant activities of the preparations were as follows: IFN- $\beta$ ,  $6.3 \times 10^6$  units/mg protein, IFN- $\gamma$   $3.2 \times 10^5$  units/mg protein. Murine IFN- $\alpha\beta$  international standard G002-904-511 was obtained from National Institute for Health, Bethesda, Maryland, USA.

#### (F) Antibodies

1. Murine monoclonal anti-H2K<sup>k</sup> (IgG<sub>2a</sub>) was provided by Dr D J Maudsley (University of Warwick) in both supernatant and ascites form derived from hybridoma clone 11-4-1 (ATCC TIB95). These preparations were used neat and at a 1:100 dilution, respectively.
2. Murine monoclonal anti-H2D<sup>k</sup> (IgG<sub>2a</sub>) was provided as purified antibody derived from hybridoma clone 15-3-55 (ATCC HB24) by E Culbert (ICI, Macclesfield, UK) and was used at a 1:40 dilution.
3. Murine monoclonal anti-H2A<sup>k</sup> (IgG<sub>2a</sub>) was provided as purified antibody by Mr M J Blackman (currently at National Institute for Medical Research, London) and in supernatant form from Dr D J Maudsley (University of Warwick). Both preparations were produced from

hybridoma clone 10.3.6 (ATCC TIB92) and were used at a 1:80 and 2:3 dilution, respectively.

4. Murine monoclonal anti-H2D<sup>k,k</sup>, cross-reactivity H2K<sup>b</sup> (IgG<sub>2a</sub>) was obtained from Uniscience Ltd as ascites fluid produced from hybridoma clone 3-83F and was used at a 1:20 dilution.
5. Murine monoclonal anti-H2A<sup>b</sup> (IgM) was obtained from Uniscience Ltd as ascites fluid produced from hybridoma clone 28-16-8, and was used at a 1:40 dilution.
6. Murine monoclonal anti-H2D<sup>d</sup>, cross reactivity H2D<sup>b</sup> (IgM) was produced by myself in supernatant form from hybridoma clone 28-11-5S (ATCC HB19) as described in methods section I, and was used neat.
7. Murine monoclonal anti-H2K<sup>d</sup> (IgM) was obtained from Uniscience Ltd as an ascites fluid derived from hybridoma clone 31-3-4S, and was used at a 1:50 dilution.
8. A polyclonal rabbit antiserum to cow glial fibrillary acidic protein (GFAP) was obtained from Dako Ltd, and was used at a 1:100 dilution.
9. A polyclonal goat antiserum to rat fibronectin was obtained from Cambridge Bioscience Ltd, and was used at a 1:100 dilution.
10. A polyclonal rabbit antiserum against SFV was prepared in this laboratory by C Sutton (University of Warwick). The SFV used to immunise rabbits was grown in BHK cells (see methods section A) and was purified by sucrose gradient centrifugation. Rabbits were immunised at

regular intervals by conventional techniques using Freund's complete and incomplete adjuvants. For indirect immunofluorescence staining of cell-surface SFV antigens the antiserum was diluted 1:20 and preabsorbed for 1 hour at 4°C with approximately  $1 \times 10^6$  cells of the appropriate type, to reduce non-specific binding prior to use.

11. A polyclonal rabbit antiserum to murine natural IFN- $\alpha\beta$  was produced in this laboratory by C Sutton (University of Warwick). The IFN- $\alpha\beta$  used to immunise rabbits was produced by infection of L929 cells with Newcastle Disease Virus. Cell supernatants were harvested at 24 hours post-infection, extensively dialysed against pH 2 glycine buffer to inactivate live virus and the IFN- $\alpha\beta$  partially purified by affinity chromatography on phenyl-borate-agarose-30 matrix gel. Rabbits were immunised at 14 day intervals by conventional techniques using Freund's complete and incomplete adjuvants. Rabbits were bled from the ear and the blood allowed to clot overnight at 4°C, after which the serum was clarified by centrifugation and heat-inactivated at 56°C for 30 minutes to remove complement, prior to storage at -20°C.
12. Rat monoclonal anti-murine IFN- $\gamma$  (IgG<sub>1</sub>) was produced by myself in ascites form (see methods section I) from hybridoma clone R4-6A2 which was generously provided by E Havell (Trudeau Institute, New York, USA).
13. Fluorescein isothiocyanate (FITC) conjugated goat-anti-mouse immunoglobulins (anti-IgA, IgM, IgG, heavy and light chains specific) IgG fraction was obtained from Cappel and used at a 1:80 dilution.
14. FITC conjugated sheep-anti-rabbit (anti-IgG, IgM, IgA) was obtained from Wellcome diagnostics and used at a 1:100 dilution.

15. FITC conjugated rabbit-anti-goat (anti-IgG) IgG fraction, was obtained from Nordic Immunological reagents and used at a 1:100 dilution.

#### (G) Radiochemicals

All radiochemicals used were obtained from Amersham radiochemicals plc.

1.  $^{51}\text{Cr}$ -sodium chromate (250-500 mCi/mg Cr).
2. Methyl- $^3\text{H}$ -thymidine (25 Ci/mmol).
3. 5- $^3\text{H}$ -uridine (25-30 Ci/mmol).

#### (H) Other materials

Actinomycin D (obtained from Sigma).  
Aluminium foil (obtained from Alcan Ltd).  
Ammonium chloride (obtained from Fisons plc).  
Calcium chloride (obtained from Fisons plc).  
Calibrite beads (obtained from Becton-Dickinson Ltd).  
Concanavalin A (obtained from Miles-Yeda Ltd).  
DEAE dextran (obtained from Sigma).  
Deoxyribonuclease I (obtained from Sigma).  
Dialysis tubing 8/32", 16/32" (obtained from Fisons plc).  
Diethyl-ether (obtained from May and Baker Ltd).  
Dimethyl sulphoxide (obtained from Fison plc).  
Disodium hydrogen phosphate (obtained from BDH).  
Eppendorf tubes 0.75 and 1.5 ml (obtained from Sarstedt Ltd).  
Ethanol (obtained from James Burroughs Ltd).  
Filter paper 12.5 cm analytical grade (obtained from Whatman Ltd).

Flow pore 0.45  $\mu$ m filter sterilizing units (obtained from Flow Laboratories Ltd).

Freezing ampoules 2 ml (obtained from Flow Laboratories Ltd).

Gelatin (obtained from Difco Ltd).

Glass filter paper (obtained from Whatman Ltd).

Glycerol (obtained from Fisons plc).

Glycine (obtained from Fisons plc).

Hydrochloric acid (obtained from Fisons plc).

Hypodermic syringes 21, 25 and 26 $\frac{1}{2}$  gauge (obtained from Sabre International).

Indomethacin (obtained from Sigma).

Lymphoprep (obtained from Nyegaard UK).

Lympholyte M (obtained from Sera-lab).

Kodak aktachrome 160 tungsten film (obtained from Kodak).

Magnesium chloride (obtained from Fisons plc).

Mitomycin C (obtained from Sigma).

Micropipette tips, 200  $\mu$ l and 1 ml (obtained from L.I.P. Equipment and Services).

Microtitre 96 well trays flat-bottomed (obtained from Costar Ltd).

Microtitre 96 well trays round-bottomed (obtained from Flow Laboratories).

Microtitre 96 well tray adhesive sealers (obtained from Flow Laboratories).

Multipipette tips, 200  $\mu$ l (obtained from Alpha Laboratories).

Neutral red (obtained from Difco Ltd).

Neocofilm (obtained from Nippon Shoji Ltd).

Noble agar (obtained from Difco Ltd).

Paraffin wax (obtained from BDH Ltd).

Paraformaldehyde (obtained from BDH Ltd).

Potassium chloride (obtained from Fisons plc).

Potassium dihydrogen phosphate (obtained from BDH Ltd).

4 cm Plastic test-tubes (obtained from Sterilin).  
Plastic bijous and universals (obtained from Sterilin).  
 $\beta$ -Propiolactone, grade II (obtained from Sigma).  
Rabbit complement, Pelfreez (obtained from Northeast Biomedical).  
Recombinant Interleukin-2, glycosylated (obtained from Genzyme).  
Scintillation fluid, ready value and E.P. (obtained from Beckman Ltd).  
Scintillation tubes, 6 ml (obtained from LKB Pharmacia Ltd).  
Sodium chloride (obtained from May and Baker Ltd).  
Sodium hydroxide (obtained from BDH Ltd).  
Silica (obtained from Sigma).  
Syringes 1,2,5,10 and 20 mls (obtained from Sterilin).  
2,6,10,14-Tetramethylpentadecane, 'Pristane' (obtained from Sigma).  
Tissue culture petri-dishes 6 cm (obtained from Becton Dickinson).  
24 Well-tissue culture plates (obtained from Costar Ltd).  
Trichloroacetic acid (obtained from May and Baker Ltd).  
Tris(hydroxymethyl)aminomethane, 'TRIS' (obtained from BDH Ltd).  
Triton X-100 (obtained from BDH Ltd).

#### (I) Buffers and solutions

1. Phosphate buffered saline (PBS) pH 7.4:

- 137 mM Sodium chloride
- 2.7 mM Potassium chloride
- 8 mM Disodium hydrogen phosphate
- 1.5 mM Potassium dihydrogen phosphate

2. Glycine buffer pH 2.0

- 100 mM Glycine
- Concentrated hydrochloric acid added to pH 2.0

3. 50 mM Tris-HCl pH 7.25

50 mM Trishydroxymethylaminomethane (TRIS)

Concentrated hydrochloric acid added to pH 7.25

4. 3% Paraformaldehyde solution in PBS

3 g paraformaldehyde was dissolved in 100 ml PBS by stirring at 80°C (in fume cupboard). While constantly stirring 10 µl 1M calcium chloride and 10 µl magnesium chloride was added, after which the solution was cooled to room temperature, the pH checked to be 7.4 and filter sterilized through a 0.45 µm pore. The solution was divided into aliquots and stored at -20°C.

5. Ammonium chloride red blood cell lysis solution

155 mM Ammonium chloride

1 M NaOH added to pH 7.0 and solution sterilized by autoclave

(J) Name and address of suppliers

Alcan Ltd: 90 Ashbridge Road, Chesham, Bucks, HP5 2QE, UK.

Alpha Laboratories Ltd: 40 Parham Drive, Eastleigh, Hampshire, UK.

Amersham International plc: Lincoln Place, Aylesbury, Buckinghamshire, UK.

ATCC: 12301 Parklawn Drive, Rockville, Maryland, USA.

Bantin and Kingman: The Field Station, Grimston, Aldbrough, UK.

BDH Chemicals Ltd: Fourways, Atherstone, Warwickshire, UK.

Beckman-RIIC Ltd: Progress Road, High Wycombe, Buckinghamshire, UK.

Becton-Dickinson Ltd: Between Towns Road, Cowley, Oxford, UK.

Cambridge Bioscience: 42 Devonshire Road, Cambridge, UK.

Cappel: West Chester, PA, 19380, USA.

Costar Ltd: PO Box 94, 1170 AB Badhoevedorp, Netherlands.

Dako Ltd: 22 The Arcade, High Wycombe, Buckinghamshire, UK.

Difco Ltd: PO Box 148, East Molesey, Surrey, UK.

Fisons plc: Bakewell Road, Loughborough, UK.

Flow Laboratories Ltd: Woodcock Mill, Rickmansworth, Hertfordshire, UK.

Genzyme Corporation: 75 Kneeland Street, Boston, Massachusetts, USA.

Gibco Ltd: Trident House, PO Box 35, Paisley, UK.

Glaxo Laboratories Ltd: Greenford Road, Greenford, Essex, UK.

James Burroughs F.A.D. Ltd: 70 Eastways Industrial Estate, Witham, Essex, UK.

Kodak Photographic Ltd: PO Box 33, Swallowdale, Hemel Hempstead, Hertfordshire, UK.

Lee Biomolecular: 11211 Sorrento Valley Road, San Diego, California, USA.

L.I.F. Equipment and Services: 111 Dockfield Road, Shipley, Yorkshire, UK.

LEB Pharmacia Ltd: Midsummer Boulevard, Milton Keynes, Buckinghamshire, UK.

May and Baker Ltd: Liverpool Road, Eccles, Manchester, UK.

Miles-Yeda Ltd: Box 37, Stoke Poges, Slough, Berkshire, UK.

Nippon Shoji Kaisha Ltd: Osaka, Japan.

Nordic Immunological Reagents: PO Box 544, Maidenhead, Berkshire, UK.

North-East Biomedical: PO Box 45, Uxbridge, UK.

Northumbria Biologicals Ltd: South Nelson Industrial Estate, Cramlington, Northumberland, UK.

Nyegaard Ltd: 11 Wagon Lane, Sheldon, Birmingham, UK.

Olac Ltd: Shaws Farm, Bicester, Oxon, UK.

Sabre International: Manor Farm Road, Reading, UK.

Sarstedt Ltd: 68 Boston Road, Beaumont Leys, Leicester, UK.

Sera-lab: Crawley Down, Sussex, UK.

Sigma London Chemical Co. Ltd: Fancy Road, Poole, Dorset, UK.

Sterilin Ltd: 43 Broad Street, Teddington, Middlesex, UK.

Uniscience Ltd: 12 St Annes Crescent, London, UK.



Wellcome Diagnostic: Temple Hill, Dartford, UK.

Whatman Ltd: Unit 1, Coldred Road, Parkwood, Maidstone, UK.

## Methods

### (A) Production and assay of SFV

All of the SFV used during the course of this project for the *in vitro* infection of cells, was of the virulent L10 strain. Laboratory stocks of virus were prepared by a single passage of seed stock mouse brain SFV (produced by passage of virus in newborn mouse brains by Mr M J Blackman, currently at National Institute for Medical Research, London) in BHK-21 cells. More specifically, BHK-21 cells were grown to confluency in Winchester roller bottles containing 100 ml GEM BHK supplemented with 10% FCS. The medium was then replaced with 40 ml GEM BHK supplemented with 2% FCS and 20  $\mu$ l seed mouse brain SFV added. The cells were then incubated for 1 hour at 37°C to allow virus to adsorb. The medium was then replaced with 100 ml of GEM BHK supplemented with 2% FCS, and the roller bottles gassed with 5% carbon dioxide in air before incubation at 37°C for 30 to 36 hours, after which a cytopathic effect was observed. Cell supernatants were then harvested, cell debris pelleted by centrifugation for 10 minutes at approximately 4,000 rpm in an MSE minor bench centrifuge, and the pH of supernatants containing SFV adjusted to pH 7.0 with bicarbonate prior to storage in aliquots at -70°C. Infectivity of the stock SFV was assessed by incorporation of [<sup>3</sup>H]-uridine into viral RNA in infected cells (see methods section F) and by plaque assay. For plaque assay, ten-fold dilutions of virus were made in ice-cold PBS (containing 0.5% FCS) and 200  $\mu$ l aliquots inoculated in duplicate onto monolayers of chick embryo fibroblasts in 6 cm petri-dishes (obtained from Prof N Dimmock, University of Warwick). After allowing the virus to adsorb for 1 hour at 33°C in a Wadco incubator with an atmosphere of 5% CO<sub>2</sub> in air, monolayers were overlaid with 4 ml 199 medium containing 2% FCS, 0.9% noble agar and 0.04% DEAE dextran. The

petri-dishes were then incubated for 2 days at 33°C as before. Plaques were visualised by staining the monolayer with 3 mls 0.01% neutral red in PBS for 3 hours at 33°C, and counted. The titre of stock virus was usually around 10<sup>9</sup> plaque forming units per ml (pfu/ml).

#### (B) Preparation of $\beta$ -propiolactone inactivated SFV (BPLSFV)

In order to produce a preparation of SFV which was antigenically intact yet unable to replicate in cells, stocks of virus were treated with  $\beta$ -propiolactone essentially by the method of Barrett *et al.* (1984). Stock virus was dialysed overnight against 50 mM Tris buffer pH 7.25 at 4°C, then incubated with 0.1%  $\beta$ -propiolactone for 5 hours at 4°C, followed by overnight dialysis against the same Tris buffer to remove residual  $\beta$ -propiolactone and its breakdown products, prior to two overnight dialyses back into GMEM. This procedure was found to reduce the infectivity of the SFV stock from 10<sup>9</sup> pfu/ml to less than 10 pfu/ml, rendered the virus unable to undergo replication - as assessed by the incorporation of <sup>3</sup>H-uridine into viral RNA in infected cells, and abolished the ability of the virus to produce a cytopathic effect when added to primary brain cell cultures (prepared as described in methods section F). Since defective-interfering (DI) viruses have been shown to inhibit the ability of standard virus to synthesise virus-specific RNA in infected cells or produce plaques on an infected monolayer (Barrett *et al.*, 1981; Johnston *et al.*, 1975) the possibility existed that live virus was still present in the  $\beta$ -propiolactone-inactivated SFV (BPLSFV) preparation, but that DI virus also present was inhibiting the ability to detect the live virus in the assay systems used. To investigate this possibility and determine whether any DI virus was present in the BPLSFV preparation, a plaque assay was set up in which a dilution series of live standard virus was assayed in the presence

or absence of the BPLSFV preparation. If DI virus were indeed present in the BPLSFV preparation then the ability of the live standard virus to produce plaques in the presence of this preparation should be inhibited as compared with the parallel assay in which the virus was titrated in the absence of the BPLSFV preparation. In fact no difference was observed between the number of plaques or size of the plaques obtained (data not shown) indicating that neither detectable DI virus nor live virus were present in the BPLSFV preparation.

#### (C) Inoculation of mice with virus

##### (i) Intranasal

Mice were placed under light ether anaesthesia. A 20  $\mu$ l aliquot of the A7 (74) strain of SFV, diluted in GHEM supplemented with 2% FCS, was then placed on the tip of the nose and was subsequently inhaled by the mice. For immunisation of 5 to 6 week-old male or female C3H/He, C57BL/6, A/J and B10.A.5(R) mice  $6.4 \times 10^5$  pfu of virus were administered. For immunisation of 5 to 6 week-old male or female Balb/c and DBA/2 mice  $1.6 \times 10^6$  pfu of virus were administered. For experiments in which 6 week-old male or female C3H/He mice were challenged with 10LD<sub>50</sub> of the A7(74) strain of SFV,  $6.4 \times 10^6$  pfu of virus were administered.

##### (ii) Intraperitoneal

For the intraperitoneal infection of newborn C3H/He mice the abdominal wall was first washed with ethanol and then  $8 \times 10^3$  pfu of the A7(74) strain of SFV in 50  $\mu$ l of GHEM medium were administered intraperitoneally using a 1 ml Sterilin syringe with a 26 $\frac{1}{2}$  gauge needle. For experiments in which 6 week-

old male or female C3H/He mice were infected with the L10 strain of SFV. mice were placed under light ether anaesthesia, the abdominal walls were then washed with ethanol and  $10LD_{50}$  ( $1.3 \times 10^8$  pfu) of the L10 strain of SFV in 200  $\mu$ l PBS administered intraperitoneally using a 1 ml Sterilin syringe with a 21 gauge needle.

#### (D) Administration of interferon gamma and antibodies to interferon gamma to mice.

Interferon gamma was first diluted to the appropriate concentration in PBS (see results legend for details) and placed on ice. The abdominal walls of the mice were then washed with ethanol and either 200  $\mu$ l or 500  $\mu$ l aliquots of interferon gamma administered intraperitoneally using a 1 ml Sterilin syringe and 21 gauge needle. For experiments in which an ascites fluid containing neutralising monoclonal antibodies to interferon gamma were used, exactly the same was performed except that the ascites fluid (neutralising titre  $10^5$ ; see methods section I) was always used neat and 200  $\mu$ l aliquots administered.

#### (E) Harvesting of mouse tissues

The following procedures were used to isolate serum and produce a suspension of mouse brain from which the presence of interferon, virus or antibodies to interferon gamma were investigated for experiments presented in this thesis.

#### (1) Serum

To obtain blood from newborn mice, the mice were first swabbed with ethanol

and then killed by decapitation. Blood was then collected from the severed neck into a 1.5 ml eppendorf tube on ice. Adult mice were killed by cervical dislocation, blood harvested aseptically from the heart and then placed into a 1.5 ml eppendorf tube on ice using a pasteur pipette. Both newborn and adult blood was allowed to clot overnight at 4°C and the next day serum isolated by centrifugation for two minutes at 10,000 g in an eppendorf microfuge. Serum was stored at -70°C prior to use.

#### (ii) Brain

Newborn and adult mice were killed as described above and the brains aseptically harvested then placed into plastic bijoux containing 0.75 ml or 3.5 ml of ice-cold PBS supplemented with 0.5% FCS, respectively. A suspension was produced by dispersing the brain into the PBS solution using a 1 ml Sterilin syringe. Aliquots were then stored at -70°C. When required, the brain suspensions were thawed in a waterbath at 37°C and the cell debris pelleted by centrifugation at 10,000 g for two minutes in an eppendorf microfuge. The supernatant was then harvested and assayed.

#### (7) Preparation of primary brain cell cultures from newborn C3H/He mice

Primary brain cell cultures were prepared from newborn to 2-day-old C3H/He mice. Ten to fifteen mice were killed by decapitation with sterile dissecting scissors. The heads were then washed with ethanol, the skin over the skull removed and the brain revealed by cutting the skull open from the base of the skull over towards the nose. The brains were then removed and placed in a sterile plastic universal containing 5 ml sterile PBS. The brains were then carefully rolled on dry filter paper (sterilized by 20 minutes irradiation under UV light) to remove the meninges, and the

tissue obtained placed in a 6 cm petri-dish containing 5 ml PBS and a stainless steel mesh, through which the tissue was subsequently gently disaggregated using a glass rod. The homogenate was then rinsed off the mesh, placed in a plastic universal, and the mesh washed a further 3 times with 5 ml of PBS to remove any remaining tissue which was also placed in the universal. The homogenate was then placed in a 20 ml Sterilin syringe and passed through a 21 gauge needle to further disaggregate the tissue, which was pelleted by centrifugation for 5 minutes at approximately 3,000 rpm in an MSE minor bench centrifuge, and reduced to a single cell suspension by trypsinisation with 10 ml trypsin at 1 mg/ml in PBS also containing 40  $\mu$ g/ml DNase-I (to reduce viscosity due to released DNA) and 0.2 mM magnesium chloride, for 30 minutes at 37°C. 10 ml of DMEM supplemented with 10% FCS were then added and the brain cells pelleted by centrifugation as before. After aspiration of the supernatant the brain cells were resuspended in DMEM supplemented with 10% FCS, and the number of cells counted using a Neubauer counting chamber. Around  $2 \times 10^6$  cells were obtained per brain.  $1-2 \times 10^6$  cells were then seeded onto 6 cm tissue culture petri-dishes in 5 ml DMEM supplemented with 10% FCS. The cells were then incubated at 37°C in a Wedco incubator with a humidified atmosphere of 5% carbon dioxide in air. Fresh medium was added at 2, 4 and 7 days after seeding and the cells were usually confluent between day 8 and 10. This method of preparation of brain cells is essentially that of Gates *et al.* (1983).

(G) Procedure for indirect immunofluorescence staining of intracellular glial fibrillary acid protein and fibronectin for observation by UV microscopy

Primary brain cell cultures were produced as described in the previous

section and grown until nearly confluent in 6 cm plastic tissue culture petri-dishes. 2 cm discs were then quickly cut out of the dishes using a hot cork-borer, washed twice with PBS and the cells fixed with 3% paraformaldehyde solution (see materials, section I) for 20 minutes at room temperature. The cells were then washed twice with PBS, quenched for 10 minutes with 50 mM ammonium chloride in PBS, and washed a further twice in PBS before submerging in 0.2% triton X-100 in PBS for 4 minutes to permeabilise the cells. 50  $\mu$ l aliquots of antibody to both glial fibrillary acid protein and fibronectin (each diluted 1:100 with 0.2% gelatin in PBS [gelatin/PBS]) were then placed onto a strip of parafilm lying flat on wet filter paper in a plastic box. Separate discs were placed cell side down onto each of the antibody solutions and incubated for 20 minutes at room temperature, after which the cells were washed three times in gelatin/PBS and then incubated with 50  $\mu$ l of the relevant FITC-conjugated antibody (diluted 1:100 in gelatin/PBS) as before. The cells were then washed a further three times in gelatin/PBS, twice in PBS and coverslips mounted onto the disc using a small drop of glycerol. The plastic side of the disc was then carefully washed with water to remove any remaining PBS and the cells observed under a Zeiss UV microscope. Photographs were taken using Kodak Ektachrome 160 tungsten film, exposed for 160 seconds.

(H) Indirect immunofluorescence staining of cell surface antigens and quantification by flow cytometry

The expression of SFV and MHC antigens on the surface of cells was measured by indirect immunofluorescence staining with quantification by flow cytometry.



(i) Interferon treatment of cells and staining of cell-surface antigens by indirect immunofluorescence

The cells were grown until nearly confluent in 6 cm tissue-culture petri-dishes in the appropriate medium supplemented with 10% FCS (see materials section B) in a Wedco incubator at 37°C with an humidified atmosphere of 5% carbon-dioxide in air. The medium was then aspirated and replaced with 3 ml of the same medium containing the appropriate concentration of interferon (see results legend). 3 ml of medium alone were added to control cultures. The cells were then incubated for a further 48 hours at 37°C (48 hour treatment with IFN has been shown to induce maximal levels of H-2 antigen on brain cells: Wong *et al.*, 1984) after which the medium was aspirated, the monolayer washed with 2 ml of 0.02% EDTA in PBS and 2 ml of the same solution also containing trypsin (1 mg/ml) added. The cells were then incubated for 5 minutes at 37°C (as before) to detach the cells from the monolayer. For experiments in which SFV antigens were to be stained the cells were detached by 15 minutes incubation at 37°C (as before) with 3 ml of 0.02% EDTA in PBS alone. Trypsinisation has no effect upon MHC antigen display by cells (Wong *et al.*, 1984). The cells were then transferred into a plastic universal containing 5 ml medium (as before) and were pelleted by centrifugation for 5 minutes at approximately 3,000 rpm in an MSE minor bench centrifuge. The supernatant was then aspirated and the cells resuspended in PBS supplemented with 0.1% sodium azide (PBS/azide) and placed on ice. Treatment of the cells with azide and holding on ice prevents the antibody capping at a later stage. Approximately  $5 \times 10^5$  cells in 200  $\mu$ l PBS were then transferred into the wells of a 96-well round-bottom microtitre plate. The cells were pelleted at 1,000 rpm in an MSE mark II centrifuge and the supernatants carefully discarded. The cells were then resuspended in 200  $\mu$ l of the appropriate

antibody specific for the antigen of interest. A proportion of the cells harvested from the plates for each treatment were also stained with an irrelevant antibody (whenever possible irrelevant antibody of the same class and subclass was used) in order to monitor background levels of fluorescence by the cells. Any staining above such background levels could then be attributed to the specific staining of cell surface antigens. All antibodies used were previously titrated and used in saturating concentrations (see section [iii] below). After resuspending the cells in antibody, the microtitre plates were covered with an adhesive plate sealer and placed on a microtitre plate shaker for 30 minutes at 4°C. The cells were then pelleted as before, washed twice with 200 µl PBS/azide (covering the plates as before prior to each centrifugation step) and resuspended in 200 µl of the appropriate fluorescein-isothiocyanate (FITC) conjugated antibody specific for the species of origin of the first antibody used. The cells were then shaken for a further 30 minutes at 4°C, then washed twice with 200 µl PBS/azide, resuspended in 200 µl of PBS/azide and stored at 4°C prior to analysis of fluorescence by flow cytometry. Typically cells were analysed within 24 hours, however if the samples were to be left for longer periods of time the cells were fixed with 200 µl of 3% paraformaldehyde solution (see materials section I) and washed once in PBS/azide prior to resuspension as before and storage at 4°C. For some experiments (see results legend) propidium iodide was used to stain non-viable cells, and in such cases the cells were resuspended in PBS containing propidium iodide at 1 µg/ml immediately prior to analysis. For most experiments presented in this thesis the flow cytometer analysis of cells was performed by myself, using the Becton-Dickinson FACStar flow cytometer. The flow cytometer was set-up and the laser aligned with the sample stream using calibrate beads, as described in the users manual. All fluorescence data were collected in list mode using logarithmic

amplification and analysed subsequently (see section [ii] below). For some experiments (see results legend) the Becton-Dickinson FACS440 at the University of Birmingham was used, and in such cases the machine was operated by Miss A Milner or Mr R Byrd with fluorescence data again being collected in list mode, but using linear amplification. For both the FACS440 and FACStar, forward scatter and side scatter signals were amplified by linear amplification. Between 5,000 and 20,000 cells were analysed for each sample.

#### (ii) Analysis of data

Data were analysed using the Becton-Dickinson Consort-30 computer program. Typically histograms of number of cells per channel against fluorescence intensity channel were plotted. Statistical data giving the mean fluorescence, peak channel (i.e. fluorescence channel with the largest number of cells collected in it) and coefficient of variation were also printed. To determine the proportion of cells showing specific fluorescence (hence displaying the antigen under investigation) a marker was set on the fluorescence histogram for a control sample (stained with an irrelevant antibody) at a point where only 5% of the cells processed were of higher fluorescence. For each sample thereafter the same marker was used and the proportion of cells above the marker determined. If the percentage of cells above the marker exceeded the 5% background value then this was attributed to specific staining, hence expression of the antigen of interest. Similarly, to determine the percentage of cells induced to express a particular antigen by interferon treatment, a marker was set on the fluorescence histogram of the non-interferon treated cells (stained with the antibody to the antigen of interest) in a similar manner, and the same marker used for all the interferon treated samples with the proportion

of cells above the marker determined as before. With very few exceptions, control samples stained with an irrelevant antibody always showed an identical level of fluorescence. Consequently, differences in fluorescence seen between samples stained with specific antibody were taken as being significant whenever they were observed. <sup>22</sup> A package for statistical analysis of fluorescence data is available within the Consort-30 computer program. Preliminary studies indicated that differences in fluorescence were statistically significant ( $p < 0.05$ ) whenever they were observed. The statistical package was not used for routine analysis of data.

#### (iii) Titration of antibody preparations

Preparations of antibody were titrated in order to determine the highest dilution of antibody which, when used in the above protocol for indirect immunofluorescence staining, gave maximal staining of the antigen under investigation. For titration of antibody, the appropriate cell type was prepared, treated with interferon, harvested and then stained with a dilution series of the antibody in exactly the same way as described in section (i). The cells were then stained with a saturating amount of the appropriate FITC-conjugated antibody (titrated in a previous experiment) and analysed by flow-cytometry (as before). To determine the highest dilution of antibody which gave maximal staining of the antigen (and above which the fluorescence of cells became dependant upon the dilution of antibody) histograms were plotted of fluorescence intensity channel against number of cells per channel, and correlated with the dilution of antibody used.

#### (i) Production of monoclonal antibody supernatant and ascites fluid

A supernatant containing monoclonal antibodies specific for H2D<sup>d</sup> (cross-reactivity H2D<sup>b</sup>) was prepared from the hybridoma clone HB19 (see materials section B). The hybridoma cells were cultivated in DMEM medium supplemented with 10% FCS and 1 mM sodium pyruvate, in 175 cm<sup>2</sup> tissue culture flasks incubated at 37°C in a Wedco incubator with a humidified atmosphere of 5% carbon dioxide in air. The cells grew in suspension and were subcultured several times to fresh medium at a dilution of 1 in 10, prior to growing until a heavy suspension of cells had formed and the medium

exhausted, after which the cells were pelleted by centrifugation in a plastic universal at approximately 3,000 rpm in a MSE minor bench centrifuge, and the supernatants harvested and stored at  $-20^{\circ}\text{C}$ , prior to titration and use.

To produce a preparation of ascites fluid containing monoclonal antibodies specific for murine interferon gamma, 200  $\mu\text{l}$  of the ascites inducing substance 2, 6, 10, 14-tetramethylpentadecane ('Fristane') were first administered intraperitoneally to 10 week old male or female MFI  $\text{nu}^+/\text{nu}^+$  nude mice, using a 1 ml Sterilin syringe and 21 gauge needle. 14 to 21 days later  $5 \times 10^6$  RA-6A2 hybridoma cells, cultivated as described above, were administered intraperitoneally to the mice in 500  $\mu\text{l}$  PBS (as before). After a further 14 to 21 days the mice became enlarged and were then killed by cervical dislocation and the ascites fluid aseptically removed taking care not to puncture the stomach or intestines, using a 10 ml syringe and 21 gauge needle. Cellular material was then pelleted by centrifugation at approximately 3,000 rpm in an MSE minor bench centrifuge and the ascites fluid stored at  $-70^{\circ}\text{C}$ , prior to use. Around 30% of the mice to which the hybridoma clone was administered developed solid tumours without ascites fluid, and were discarded.

The neutralisation titre of an anti-interferon gamma ascites preparation was defined as being the reciprocal of the highest final dilution of antibody which neutralised 50% of the antiviral activity of a laboratory recombinant interferon gamma preparation (titre 1,000 units/ml) when titrated in a standard interferon neutralisation assay (see methods section P). Since a linear relationship was known to exist between the amount of RA-6A2 antibody present and neutralisation of interferon-gamma antiviral activity (Havell, 1983), the neutralisation titre of the ascites fluid was

obtained by plotting a graph of dilution of ascites fluid against percent neutralisation of interferon gamma standard antiviral activity, and determining the dilution at which the line intersect with the 50% neutralisation of antiviral activity value. The neutralisation titre of the ascites preparations was always found to be within the range of  $10^3$  to  $10^6$ .

#### (J) Preparation of SFV-specific effector T-cells

Mice were immunised intranasally with the A7(74) avirulent strain of SFV as described in methods section C. At day 7 post-infection apparently healthy mice were killed by cervical dislocation and the spleens aseptically removed. Up to 4 spleens were then placed onto a stainless steel mesh in a 6 cm petri-dish containing 5 ml PBS, and converted into a single-cell suspension by gently teasing with a glass rod. The cell-suspension was then placed in a plastic universal and the mesh washed with a further 5 ml of PBS to remove remaining cells. 10 ml of ammonium chloride solution (see materials section I) was then added for 2 minutes at room temperature to remove red blood cells by osmotic lysis. The cell suspension was pelleted for 5 minutes at approximately 3,000 rpm in an MSE minor bench centrifuge, and gently resuspended in 10 ml of RPMI 1640 complete media supplemented with 10% FCS (see materials section A). Cells were counted using a Neubauer counting chamber and resuspended at  $5 \times 10^6$ /ml in the same medium containing a 1:30 dilution of  $\beta$ -propiolactone-inactivated SFV (see methods section B). Cells were then placed in a Costar 24 well plate at 1.5 ml/well, and incubated at 37°C in a Wedco incubator with an humidified atmosphere of 5% carbon-dioxide in air. Cells were harvested at day 5 post-culture for cytotoxicity assays and day 7 post-culture for proliferation and IFN- $\gamma$  release studies. Harvested cells were passed over

Lymphoprep or Lympholyte M to remove non-viable cells and debris, prior to use.

#### (K) Preparation of alloreactive effector T-cells

To generate Balb/c-anti-C3H/He ( $H2^d$ -anti- $H2^k$ ) alloreactive effector cells, three Balb/c mice were primed intraperitoneally with  $1 \times 10^8$  live C3H/He splenocytes in 500  $\mu$ l PBS (spleen cell suspensions were prepared as described in methods section J). Ten to fourteen days later single cell-suspensions were prepared from the spleens of these primed mice and also from three C3H/He mice. The C3H/He (stimulator) splenocytes were then treated with mitomycin C at 25  $\mu$ g/ml in PBS for 45 minutes in the dark at 37°C, followed by four washes in RPMI complete medium supplemented with 10% FCS (see materials section A). Treatment with mitomycin C results in the cross-linking of DNA and thereby inhibits cellular division, protein synthesis by the cells is not affected (Swain, 1980).  $25 \times 10^6$  C3H/He spleen cells were then mixed with  $25 \times 10^6$  primed Balb/c cells in 25 ml RPMI medium (as before) in a 25  $cm^2$  tissue culture flask, which was incubated standing upright at 37°C in a Wedco incubator with a humidified atmosphere of 5% carbon-dioxide in air. On day 5 post-culture 3 ml of medium was removed and replaced with the same volume of fresh medium, to prevent the medium becoming too acidic. For cytotoxicity assays and proliferation assays cells were harvested on day 5 and day 7 post-culture respectively, and passed over Lymphoprep or Lympholyte M to remove non-viable cells and debris, prior to use. Balb/c-anti-C37 BL/6 ( $H2^d$ -anti- $H2^b$ ) and C3H/He-anti-Balb/c ( $H2^k$ -anti- $H2^d$ ) alloreactive effector cells were prepared in exactly the same way but using the different strains of mice as appropriate.



(L) Harvesting of lymphocytes for assay of non-specific cytotoxicity

Mice were treated with IFN- $\gamma$ , antibodies to IFN- $\gamma$  and PBS as described in methods section D. The spleens were then removed and a single-cell suspension prepared as described in methods section J, with the exception that the cells were not subjected to ammonium chloride treatment. Approximately  $5 \times 10^7$  splenocytes in 4 ml of RPMI 1640 complete medium supplemented with 10% FCS (see materials section A) were then carefully layered onto 8 ml of Lympholyte M in a plastic universal and centrifuged at approximately 3,000 rpm for 15 minutes in an MSE minor bench centrifuge. All medium, Lympholyte M and centrifugation were at room temperature to facilitate the separation of lymphocytes. The viable lymphocytes were then removed from the interface using a pasteur pipette, pelleted by centrifugation for 5 minutes (as before) and washed once in medium (as before) prior to use. The cytotoxicity of the lymphocytes was then determined against Yac-1 cells in essentially the same way as described in methods section M.

(M) Cytotoxicity Assays

For SFV-specific cytotoxicity assays, cultures of primary brain cells, C1300 cells or G26-24 cells were grown until nearly confluent in 6 cm tissue-culture petri-dishes containing 5 ml DMEM medium supplemented with 10% FCS, and incubated at 37°C in a Wedco incubator with an humidified atmosphere of 5% carbon-dioxide in air. The cells were then treated with 3 ml of the same medium containing IFN- $\alpha\beta$  or IFN- $\gamma$  at the appropriate concentration (see results legend) and incubated as before for 48 hours prior to cytotoxicity assay. Control cultures were untreated and 3 ml medium alone added. 16 hours prior to cytotoxicity assay 50  $\mu$ Ci  $^{51}$ Cr

(sodium chromate, see materials section G) were added to each culture to allow the cells to take up  $^{51}\text{Cr}$  overnight. The next morning cells were either infected with SFV (multiplicity of infection approximately 1000) treated with an equivalent amount of BPLSFV (see methods section B) or mock infected with medium alone. At 2 hours post-infection the cells were washed three times with 2 ml medium, once with 2 ml 0.02% EDTA in PBS and the cells detached from the monolayer by incubation with 2 ml of the same EDTA solution for 15 minutes at  $37^{\circ}\text{C}$ . The cells were then harvested using a pasteur-pipette, placed into 5 ml medium in a plastic universal, pelleted by centrifugation for 5 minutes at approximately 3,000 rpm in an MSE minor bench centrifuge, resuspended in fresh medium and counted using a Neubauer counting chamber. The cell concentrations were adjusted to  $1 \times 10^5/\text{ml}$  and 100  $\mu\text{l}$  aliquots added to the wells of 96 well round bottomed microtitre plates. 100  $\mu\text{l}$  aliquots of SFV-specific effector T-cells (see materials section J) were then added to the wells at between 34 to 5 hours post-infection (see results legend) to give effector:target ratios (E:T) within the range 1:1 to 50:1. In addition, 100  $\mu\text{l}$  aliquots of 1 M hydrochloric acid or medium were added to control wells of each target cell type in order to assess total release and spontaneous release of  $^{51}\text{Cr}$  from the cells. 4 to 5 hours later (see results legend) the microtitre plate was sealed with an adhesive plate sealer and subjected to centrifugation at 1,000 rpm for 1 minute to pellet cells and facilitate dissolution of  $^{51}\text{Cr}$  from lysed cells, after which 100  $\mu\text{l}$  of each supernatant was removed and placed in a 4 mm plastic test-tube which was sealed with paraffin wax.  $^{51}\text{Cr}$ -released was determined using an LKB gamma counter and specific release calculated using the formula:

$$\% \text{ lysis} = \frac{(\text{release in presence effectors} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} \times 100$$

In each experiment cytotoxicity of the effector T-cells against SFV-infected lymphoblastoid or fibroblast cells was assessed in order to monitor the specificity and restriction of killing. Cytotoxicity against Yac-1 cells (a cell line highly susceptible to natural killer cell lysis: Chervenak and Wolcott, 1988) was also assessed to monitor levels of natural killer cell activity in the effector population.

The susceptibility of the target cells to lysis by anti-SFV in the presence of complement was also assessed in parallel for each assay, in order to monitor levels of viral antigen display. Here, 35 minutes before harvesting the virus-specific T-cell assay, either 50  $\mu$ l aliquots of a 1:30 dilution of anti-SFV antibody in PBS or PBS alone were added to a duplicate microtitre plate (containing 100  $\mu$ l aliquots of each cell type) which was then incubated for 30 minutes at 4°C. 50  $\mu$ l aliquots of a 1:10 dilution of rabbit complement in PBS were then added to the wells, prior to incubation for a further 30 minutes at 37°C, after which supernatants were harvested and <sup>51</sup>Cr-released assessed as before. In addition, control wells to assess total release and spontaneous release values were set up for this assay as before.

Alloreactive cytotoxicity assays were carried out in essentially the same way as for virus-specific cytotoxic T-cell assays except that the SFV infection was omitted, cells were harvested using 0.02% EDTA in PBS containing 1 mg/ml trypsin (trypsinisation has no effect upon MHC antigen expression by cells; Wong *et al.*, 1984) and alloreactive effector T-cells were used (see methods section K).

Natural cytotoxicity assays were again carried out in essentially the same way as for virus-specific assays, except that the SFV infection was omitted and Yac-1 cells were used as target cells. Effector lymphocytes were prepared as described in methods section L.

(M) Assay to study the ability of SFV-treated astrocytes to stimulate SFV-specific T-cells to proliferate and release IFN- $\gamma$

In order to determine whether interferon pretreatment of astrocytes, prior to treatment with a  $\beta$ -propiolactone inactivated preparation of SFV (BPLSFV), modulates the ability of the cells to stimulate SFV-specific T-cells to proliferate, an assay was set up in essentially the same way as for SFV-specific cytotoxic T-cell assays with the exception that; astrocytes were not labelled with  $^{51}\text{Cr}$ : astrocytes were treated with mitomycin C at 25  $\mu\text{g}/\text{ml}$  in PBS for 1 hour at 37°C in the dark followed by five washes with RPMI 1640 complete medium supplemented with 10% FCS, the SFV-specific effector T-cells used (prepared as described in methods section J) were depleted of accessory cells by incubation with 200  $\mu\text{g}/\text{ml}$  silica in RPMI 1640 medium (as before) for 24 hours prior to harvesting over Lymphoprep and addition to the assay, and BPLSFV at a final dilution of 1:4 was added to each well. The co-cultures of astrocytes with SFV-specific T-cells were then incubated for 48 hours at 37°C in a Wadco incubator with a humidified atmosphere of 5% carbon-dioxide in air, after which 20  $\mu\text{l}$  of medium (as before) containing 1  $\mu\text{Ci}$  tritiated thymidine were added to each well. The cells were then incubated a further 24 hours at 37°C (as before) and harvested onto glass filter paper using a MASH II cell harvester. The filter paper was then allowed to dry at room temperature and discs containing the harvested cells cut out and placed into plastic

scintillation vials. 3 mls of scintillation fluid was added and the amount of tritiated thymidine incorporated assessed using a Rackbeta scintillation counter.

In order to monitor the production of IFN- $\gamma$  by SFV-effector T-cells stimulated with BPLSFV treated astrocytes, an assay was set up in which astrocytes were cultured in the presence and absence of SFV-effector T-cells again in essentially the same way as for SFV-specific cytotoxic T-cell assays except that; astrocytes were not labelled with  $^{51}\text{Cr}$ , only an effector to target ratio of 10 to 1 was used, and cells were only treated with either BPLSFV or media (since live virus would interfere with the subsequent interferon assay). The supernatants were harvested from the cocultures of astrocytes with or without SFV-effector T-cells at 6 hours coculture, and were subjected to centrifugation for 2 minutes at approximately 10,000 g in an eppendorf microfuge to pellet any cells collected, after which the supernatants were harvested and interferon activity assayed and characterised by both interferon assay and interferon neutralisation assay (see methods section F). Six replicate titrations were performed for each sample, from which the mean titre and standard deviation were calculated. For experiments investigating the effect of these supernatants upon class I and class II MHC expression by astrocytes, the supernatants were diluted in DMEM supplemented with 10% FCS and added to cultures of the appropriate brain cell type in a 6 cm tissue culture petri-dish which were then incubated for 48 hours at 37°C, as before. MHC antigens were stained by indirect immunofluorescence and fluorescence quantitated by flow cytometry as described in methods section H.

#### (O) Allogproliferation assays

For allogproliferation experiments an assay was set up essentially the same way as for alloreactive cytotoxicity assays (see methods section M) except that the brain cells when harvested were washed once with 5 mls of PBS and then treated with 5 mls of mitomycin C at 25  $\mu\text{g/ml}$  in PBS for 45 minutes at 37°C in the dark. The cells were then washed four times with RPMI 1640 complete medium supplemented with 10% FCS to remove mitomycin C, prior to the addition of alloreactive responders (prepared as described in methods section K) at ratios of 3 and 10:1. The cells were then incubated for 30 hours at 37°C in a Wedco incubator with an humidified atmosphere of 5% carbon dioxide in air, after which 20  $\mu\text{l}$  of medium (as before) containing 1  $\mu\text{Ci}$  tritiated thymidine were added to each well. After a further 24 hours incubation at 37°C (as before) the cells were harvested onto glass filter paper using a MASH II cell harvester and the amount of tritiated thymidine incorporated assessed as described in methods section N.

#### (P) Interferon assay

IFN was assayed by a modified version of the 50% inhibition of nucleic acid synthesis method of Atkins *et al.* (1974) using SFV to challenge mouse L929 cells. L929 cells were grown and a single-cell suspension prepared by trypsinisation (in the same way as for subculture) as described in materials section B. The cells were then counted using a Neubauer counting chamber and the concentration adjusted to  $2 \times 10^5/\text{ml}$ , using CMEM supplemented with 10% FCS. 200  $\mu\text{l}$  aliquots were then seeded into the wells of a 96 well flat-bottomed microtitre plate (giving 40,000 cells/well) and the plates incubated overnight at 37°C in a Wedco incubator with an humidified

atmosphere of 5% carbon dioxide in air, after which the cells had formed confluent monolayers at the base of each well. The medium was then aspirated from the wells and replaced with 180  $\mu$ l of GMEM medium containing 2% FCS (maintenance medium). 20  $\mu$ l aliquots of IFN sample were then placed in replicate wells (see results legend for number of replicate wells used) and were thoroughly mixed with the medium prior to transferring 20  $\mu$ l aliquots to the next well across the plate. This procedure was repeated giving a serial ten-fold dilution of the IFN sample across the plate. The cells were then incubated for 16 to 18 hours at 37°C (as before) to allow the IFN to induce an antiviral state in the cells. The medium was then aspirated from each of the wells and the cells challenged with 100  $\mu$ l of SFV (multiplicity of infection approximately 300) in maintenance medium also containing 3  $\mu$ g/ml actinomycin D (AMD, to inhibit cellular DNA dependant RNA synthesis but allow viral RNA-dependent RNA synthesis to continue). The cells were then incubated for 3 hours at 37°C (as before) after which 100  $\mu$ l aliquots of tritiated uridine (<sup>3</sup>H-uridine at 10  $\mu$ Ci/ml in maintenance medium containing 3  $\mu$ g/ml AMD) were added. After a further 3 hours incubation at 37°C (as before) the medium was aspirated and the plate flooded twice with ice-cold 5% trichloroacetic acid and once with distilled water. The plates were dried in an oven at 65°C and 50  $\mu$ l of 1 M sodium hydroxide added to each well. The plates were then sealed with Nascofilm and incubated overnight at 37°C in a warm room to dissolve the monolayer. The next day 50  $\mu$ l aliquots of 1 M hydrochloric acid were added to each well and the entire contents of each well transferred to plastic scintillation vials. 3 ml scintillation fluid were then added and the incorporation of tritiated uridine assessed by counting samples in an LKB Rackbeta scintillation counter for a period of 1 minute per vial. Uninfected and infected control cultures without added IFN ("cell control" and "virus control", respectively) were included in each assay to determine

the background level of tritiated uridine incorporation by AMD treated cells and the level of maximal tritiated uridine incorporation into viral RNA in infected cells. Furthermore, a laboratory IFN standard was included in order to monitor the sensitivity of each assay. The laboratory IFN- $\alpha\beta$  standard was a hundred fold dilution of the natural IFN- $\alpha\beta$  stock purchased from Lee Biomolecular and the laboratory IFN- $\gamma$  standard was a preparation of recombinant IFN- $\gamma$  (see materials section E for details of these preparations). The titre of both preparations was calibrated against National Institutes of Health murine IFN- $\alpha\beta$  standard G002-904-311, there is at present no international standard for murine IFN- $\gamma$ .

For IFN neutralisation experiments the assay was set up in essentially the same way as for a typical IFN assay except that prior to titration of the IFN samples 160  $\mu$ l of maintenance medium were added to each well, and also 20  $\mu$ l of anti-IFN- $\alpha\beta$  or anti-IFN- $\gamma$  antibody (at 1:10 and 1:50 dilution of polyclonal serum and ascites fluid, respectively). The samples were then titrated as usual in the presence of the antibodies. Furthermore, control cultures of titrations of IFN- $\alpha\beta$  standard preparations against each anti-IFN antibody solution were included, as well as titrations of each standard preparation alone, in order to monitor the specificity of neutralisation.

In this assay system 1 unit/ml of IFN is defined as the concentration of IFN which induces 50% inhibition of incorporation of tritiated uridine into virus-specific RNA in treated cells. The titre of an IFN sample can thereby be obtained by taking the reciprocal of the dilution of the IFN-sample which gave a 50% inhibition of incorporation of tritiated uridine into virus-specific RNA, hence a concentration of 1 unit/ml. To determine the 50% inhibition of incorporation value the mean counts per minute (CPM) values for the cell control and virus control wells were substituted into



the following formula:

$$50\% \text{ inhibition value} = \left[ \frac{\text{virus control CPM} - \text{cell control CPM}}{2} \right] + \text{cell control}$$

The ratio of virus control value to cell control value were typically within the range 8:1 to 25:1.

A graph of mean CPM against  $\log_{10}$  dilution factor of the sample was then plotted for each sample titrated. The IFN titre was obtained by taking the reciprocal of the dilution factor where the resulting titration curve intersected the 50% inhibition of incorporation value.

In order to determine the reproducibility and variability of an IFN titre obtained from this assay a preparation of IFN- $\gamma$  was titrated in eleven separate assays and the geometric mean and standard deviation of the titre were calculated. The mean titre was found to be 3.05 with a standard deviation of 0.12. Since the distribution of titres follows a normal distribution the 95% confidence limits for the assay can be obtained using the formula:

$$\mu \pm 1.96 \sigma = 3.05 \pm 0.24$$

Where  $\mu$  is the mean and  $\sigma$  the standard deviation of the titres (Parker, 1979). The 95% confidence limits, that is the limits between which the titre of a sample may fall in 95% of all assays in which it is titrated, are  $\pm 0.24 \log_{10}$  units. This means that as a general rule of thumb differences in  $\log_{10}$  IFN titre of greater than 0.48 units, or a three fold difference in arithmetic units, represent a statistically significant

difference in titres. Furthermore, should the IFN standard in any assay fall outside the limits  $\pm 0.24 \log_{10}$  units of the mean titre, the assay was considered to be less sensitive than usual and was repeated. It should also be noted that these confidence limits assume a constant standard deviation in titre obtained from the assay and are thereby really of use only when comparing titres obtained from titrations done in duplicate, hence from which no reliable estimate for the standard deviation can be obtained. For some experiments where comparison between titres was of particular importance each sample was titrated in six replicate titrations and the mean and standard deviation of the titre calculated. In such cases the significance of differences between titres obtained were determined using the Students *t*-test (Parker, 1979).

#### (Q) Determination of relative sensitivity of brain cells to interferon

To determine the relative sensitivity to IFN- $\alpha\beta$  and IFN- $\gamma$  of SFV replication in each of the brain cell types used during this project, the cells were distributed (in the appropriate media, see materials section B) into the wells of a 96 well flat-bottom microtitre plate, in the same manner as for a typical interferon assay. The fibroblast cell line L929, known to be highly sensitive to both IFN- $\alpha\beta$  and IFN- $\gamma$  was then distributed in parallel wells to allow comparison of sensitivity to IFN. The next day a preparation of IFN- $\alpha\beta$  or IFN- $\gamma$  (whose titre had been previously determined) was added to the first wells across the plate and a ten-fold dilution series of IFN prepared across the plate as usual. Thereafter, all procedures used were as for a typical IFN assay.

#### (3) Storage of cells in liquid nitrogen

Stocks of cell lines used during the course of this project were stored frozen in liquid nitrogen until required. For storage, cells were harvested in exactly the same way as for subculture (see materials section B) and resuspended to approximately  $1 \times 10^6$ /ml in medium containing 10% FCS and 10% dimethyl sulphoxide (medium solution was filter sterilized using a flowpore filter unit). 1 ml aliquots of cell suspension were then placed into freezing ampoules, which were wrapped in several layers of tissue paper and aluminium foil (to ensure a slow decrease in temperature) and placed at  $-20^{\circ}\text{C}$  for 2 hours,  $-70^{\circ}\text{C}$  overnight and then stored in liquid nitrogen. When required, cells were thawed quickly at  $37^{\circ}\text{C}$  in a waterbath, added to 10 mls medium in a plastic universal, pelleted by centrifugation for 5 minutes at 3,000 rpm in an MSE minor bench centrifuge and resuspended in fresh medium prior to seeding to fresh tissue culture flasks as usual. Cells were maintained in continuous culture for at least 14 days before use.

## Chapter 3

### Replication of SFV in brain cells

#### Introduction

The ability of the L10 strain of SFV to undergo replication in brain cells has been subject to a number of studies both in vivo and in vitro. Thus, electron microscopic studies have revealed the destruction of neurons within the brains of SFV-infected mice (Pathak et al., 1976) and the presence of replicating virus within both neurons (Pathak et al., 1976; Barrett et al., 1980) and oligodendrocytes (Pathak and Webb, 1983). Similarly, in vitro studies have demonstrated the ability of SFV to undergo replication in both G26-24 oligodendroglioma cells and C1300 neuroblastoma cells (Atkins and Sheahan, 1982; Atkins, 1983) as well as in primary cultures of neurons and primary cultures of brain cells which were predominantly astrocyte in composition (Gates et al., 1985).

One of the major aims of this thesis is to investigate the ways in which IFN treatment of brain cells modulates both the susceptibility of brain cells to infection by SFV, and ability to participate in SFV-specific T-cell mediated immune reactions. In this chapter the production and partial characterisation of primary brain cell cultures is described, and the ability of SFV to undergo replication in brain cell cultures, G26-24 oligodendroglioma and C1300 neuroblastoma cells investigated. Since the ability of SFV to undergo replication in each of these cell types has previously been subject to a small number of studies, comparisons of results obtained will also be made as appropriate.

## Results

### Production and partial characterization of primary brain cell cultures prepared from the brains of newborn C3H/He mice.

Primary brain cell cultures were prepared from newborn C3H/He mice as described in methods section F. Photograph 3.1 shows a typical culture of brain cells when viewed by phase contrast microscopy on day 8 post-culture, at which time the cells were usually confluent, or were approaching confluency. It can be seen that the majority of cells present were morphologically similar and resemble the cultures of astrocytes as prepared by Tedeschi and coworkers (Tedeschi *et al.*, 1986). To determine the actual proportion of astrocytes present, cells were permeabilized and glial fibrillary acidic protein (GFAP; an intracellular cytoskeletal protein marker of astrocytes; Signami *et al.*, 1972) stained by indirect immunofluorescence. Photograph 3.2a shows a culture of brain cells when stained for GFAP and viewed under ultra-violet light. It should be noted that photographs 3.1 and 3.2 were taken of cultures of brain cells prepared in an identical manner, but in separate experiments. It can be seen (Photograph 3.2a) that a large number of GFAP<sup>+</sup> astrocytes are present in these cultures and that the fluorescence is associated with an intracellular filamentous network. This pattern of staining resembles that observed previously by other workers whom stained GFAP within astrocyte cultures by indirect immunofluorescence (Fontana *et al.*, 1972; Giulian and Lachman, 1985; Tedeschi *et al.*, 1986). Observation of the pattern of GFAP staining also revealed the predominant astrocyte type present to be the type 1 protoplasmic astrocyte, an example of which is shown in photograph 3.2b. Type 2 astrocytes as determined by their smaller cell body and extended processes (see Photograph 3.2c) were rarely detected. In a series

Photograph 3.1.

Primary brain cell cultures prepared from the brains of newborn mice C3H/He  
mice



- (i) 8 day-old primary brain cell culture, phase contrast, magnification 1600 X.
- (ii) Photograph taken and printed by Mr R Ling (University of Warwick).

Photograph 3.2.

Primary brain cell culture stained with antiserum to glial fibrillary  
acidic protein by indirect immunofluorescence

(a)



(b)



(c)



Kay

(a) 8-day old primary brain cell culture stained with rabbit-anti-GFAP serum and FITC-conjugated goat-anti-rabbit serum, as described in methods section G. Viewed under ultra-violet light, magnification 1600 X.

(b) A type 1 astrocyte, stained and viewed as for (a).

(c) A type 2 astrocyte, stained and viewed as for (b).

- Photographs taken with assistance from Mr R Ling (University of Warwick).

of three experiments by myself and one experiment by Mr M J Blackman (University of Warwick) the proportion of GFAP<sup>+</sup> cells present in the brain cell cultures was found to be between 90% and 100% of the total. In each experiment the permeabilized cells were also stained by indirect immunofluorescence for fibronectin, a marker protein of fibroblasts. However fibroblasts, as determined by their reaction with the anti-fibronectin sera, were rarely found (data not shown). Since GFAP<sup>+</sup> astrocytes were by far the most predominant cell type present in these brain cell cultures, the cultures will hereafter be referred to as astrocyte cultures. It is however important to note that for each experiment within this thesis in which astrocytes are used, a small population (up to 10%) of other uncharacterised cells may also have been present.

#### Replication of SFV in astrocytes, G26-24 and C1300 cells

In order to determine whether SFV was able to undergo replication in astrocytes, G26-24 and C1300 cells, the synthesis of SFV-RNA in infected cells was monitored via the incorporation of tritiated uridine, and the display of viral antigen on the cell surface measured by indirect immunofluorescence staining with quantitation by flow cytometry.

The data in table 3.1 show the incorporation of [<sup>3</sup>H]uridine into astrocytes as compared with L929 cells, a fibroblast line derived from the C3H/He mouse known to be sensitive to infection by SFV (Meager, 1987). It can be seen that viral RNA synthesis took place in both astrocytes and L929 cells, since the amount of [<sup>3</sup>H]uridine incorporated into each of these cell types when infected with SFV, was far higher than the background level of incorporation of [<sup>3</sup>H]uridine into cellular RNA in uninfected cells.



Table 3.1

RNA synthesis by SFV in astrocytes and 1929 cells

<u>Cell type</u>		<u>Total RNA synthesis (cpm)</u>
Astrocyte	Infected	37,037 $\pm$ 3,298
	Uninfected	587 $\pm$ 139
1929	Infected	91,429 $\pm$ 8,971
	Uninfected	3,071 $\pm$ 611

- (1) RNA synthesis measured by incorporation of [<sup>3</sup>H]uridine between 3 and 6 hours post-infection, in the presence of 3  $\mu$ g/ml actinomycin D (AMD) to inhibit cellular RNA synthesis, as described in methods section F.
- (ii) Uninfected cells give a measure of residual incorporation of [<sup>3</sup>H]uridine into cellular RNA.
- (iii) Data expressed as mean counts per minute (cpm) incorporated  $\pm$  standard deviation for six replicate wells.

Similarly, the amount of [ $^3$ H]uridine incorporated into both SFV-infected G26-24 and C1300 cells was far higher than the background level of incorporation into uninfected cells (Table 3.2a and b) indicating that viral RNA synthesis had taken place in the cells. Over a time course in which incorporation of [ $^3$ H]uridine was followed, SFV infected G26-24 cells were found to incorporate far lower amounts of [ $^3$ H]uridine than SFV infected L929 cells (Table 3.2a). Similarly, SFV infected astrocytes also incorporated slightly lower amounts of [ $^3$ H]uridine than SFV infected L929 cells (Table 3.1) but in contrast, SFV infected C1300 cells incorporated slightly higher amounts of [ $^3$ H]uridine than SFV infected L929 cells (Table 3.2b).

Uninfected and SFV-infected astrocytes, G26-24 and C1300 cells were also stained with anti-SFV serum by indirect immunofluorescence and analysed by flow cytometry. As shown in figure 3.1, the fluorescence of SFV-infected astrocytes was markedly higher than for uninfected astrocytes, indicating that SFV-infected astrocytes display SFV antigen on the cell surface. Statistical analysis of the fluorescence data also revealed a shift in mean fluorescence from 7 for uninfected cells to 110 for SFV infected cells. Furthermore, the proportion of cells collected above fluorescence channel 9 was found to increase from 10% for the uninfected astrocytes, to 96% for the SFV infected astrocytes. Thus, at least 86% of the SFV infected astrocytes display SFV antigen on the cell surface. The fluorescence of both SFV infected G26-24 and C1300 cells was also found to be markedly higher than for uninfected cells (Figure 3.2a and b) indicating that the SFV-infected cells display SFV antigen on the cell surface. Statistical analysis of the fluorescence data for uninfected as compared with SFV-infected G26-24 cells, revealed a shift in mean fluorescence from 23 to 141, and an increase in the proportion of cells collected above

Table 3.2a

RNA synthesis by SFV in G26-24 and L929 cells

<u>Cell type</u>		<u>Total RNA synthesis (cpm)</u>
G26-24	Infected	20,145 $\pm$ 2,350
	Uninfected	1,149 $\pm$ 189
L929	Infected	145,192 $\pm$ 11,961
	Uninfected	5,590 $\pm$ 1,236

(1) Legend as for Table 3.1.

Table 3.2b

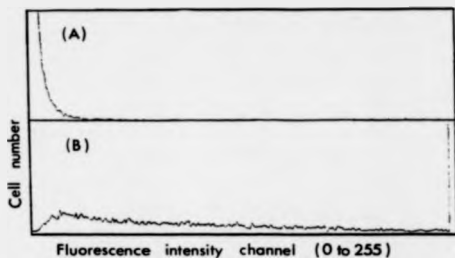
RNA synthesis by SFV in C1300 and L929 cells

<u>Cell type</u>		<u>Total RNA synthesis (cpm)</u>
C1300	Infected	122,377 $\pm$ 5,490
	Uninfected	1,991 $\pm$ 2,020
L929	Infected	82,611 $\pm$ 5,377
	Uninfected	5,108 $\pm$ 635

(1) Legend as for Table 3.1.

Figure 3.1

SFV antigen expression by infected astrocytes



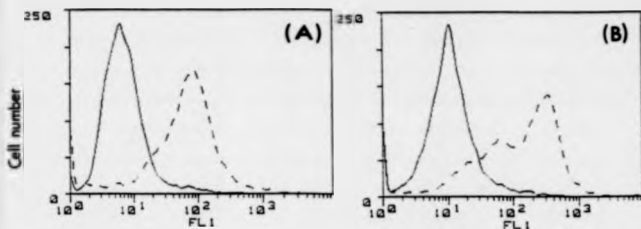
Key

- (A) Astrocytes, uninfected.
- (B) Astrocytes, infected with SFV.

- (i) Astrocytes were infected with SFV, harvested and SFV antigens stained by indirect immunofluorescence at 7 hours post-infection as described in methods section H.
- (ii) SFV antigen expression was quantified using the FACS440 flow cytometer at the University of Birmingham, operated by Miss A Milner.
- (iii) 9728 cells were analysed in each case.

Figure 3.2

SFV antigen expression by infected G26-24 and C1300 cells



Key

- |                  |                                 |
|------------------|---------------------------------|
| (A) G26-24 cells | solid line : Uninfected         |
|                  | dashed line : Infected with SFV |
| (B) C1300 cells  | solid line : Uninfected         |
|                  | dashed line : Infected with SFV |

FL1 : Fluorescence intensity channel

- (i) G26-24 cells were infected with SFV, harvested and SFV antigens stained by indirect immunofluorescence at 8 hours post-infection as described in methods section H. An identical protocol was followed for C1300 cells except that cells were stained at 9 hours post-infection.
- (ii) SFV antigen expression was quantified using the FACStar flow cytometer and 10,000 cells were analysed in each case.

fluorescence channel 37 from 5% to 71%, indicating that at least 66% of SFV-infected G26-24 cells display SFV antigen on the cell surface. Likewise, analysis of the fluorescence data for uninfected as compared with SFV-infected C1300 cells, revealed a shift in mean fluorescence from 33 to 239, and an increase in the proportion of cells above fluorescence channel 63 from 5% to 66%, indicating that at least 61% of SFV-infected C1300 cells display SFV antigen on the cell surface. The increased fluorescence observed for SFV-infected astrocytes, G26-24 and C1300 cells, was specific for staining with anti-SFV serum, since the fluorescence of SFV infected cells stained with an irrelevant antibody was identical to that of uninfected cells stained with the same antibody (data not shown).

SFV-infected G26-24 cells were also found to exhibit a clear cytopathic effect (CPE) at 8 hours post-infection, at which time SFV-infected astrocytes and C1300 cells appeared normal. By 24 hours post-infection an extensive CPE was observed for all cell types, with many cells disintegrated.

### Discussion

In this chapter the partial characterisation of primary brain cell cultures, prepared from the brains of newborn C3H/He mice, has been described. Indirect immunofluorescence staining showed that the cultures were at least 90% GFAP<sup>+</sup> astrocyte in composition, with 10% or less uncharacterised cells also present. Based upon the pattern of GFAP staining as illustrated by McCance (McCance, 1984) the majority of astrocytes were identified as type 1 protoplasmic astrocytes, with few type 2 fibrous astrocytes present. Fibronectin<sup>+</sup> fibroblasts were rarely detected in these cultures. Although the identify of (at most) 10% of the cells was not defined, it does at least seem possible that they represent a mixture of neurons, oligodendrocytes and microglial cells, since these cell types have been characterised previously in primary brain cell cultures prepared from the brains of newborn mice and rats (Bruce *et al.*, 1984; Gates *et al.*, 1985). The results presented in this chapter also clearly show that the L10 strain of SFV was able to undergo RNA synthesis in the brain cell cultures, and that at least 86% of the infected cells displayed SFV antigen on the cell surface. Since at least 90% of the cells present in these cultures are astrocytes, it follows that the majority of astrocytes present must have displayed SFV antigen on the cell surface. It is thereby not unreasonable to propose that at least some of the SFV-RNA synthesis detected in these cultures may have taken place in astrocytes. The ability of the L10 strain of SFV to undergo RNA and protein synthesis in primary brain cell cultures, predominantly astrocyte in composition and prepared from newborn Balb/c mice, has also been previously reported (Gates *et al.*, 1985). It is not possible from the data presented in this chapter to determine whether SFV-RNA synthesis had taken place in the uncharacterised cells present in these cultures, or whether any of these

cells display SFV antigen on the cell surface. However, if the uncharacterised cells present were neurones or oligodendrocytes, it would be a strong possibility since Gates and coworkers have demonstrated RNA synthesis by the L10 strain of SFV in primary cultures of neurones which were 100% pure (Gates *et al.*, 1985) and Pathak and Webb demonstrated that oligodendrocytes can be infected with the L10 strain of SFV *in vivo* (Pathak and Webb, 1983). Whether SFV is able to replicate in microglial cells which may be present in these cultures is unknown.

In agreement with previous reports by Atkins (Atkins and Sheahan, 1982; Atkins, 1983) the L10 strain of SFV was also found to undergo RNA synthesis in G26-24 and C1300 cells, as assessed by the incorporation of [<sup>3</sup>H]uridine into infected cells. When compared to the amount of SFV-RNA synthesised in L929 cells, the amount of SFV-RNA synthesised in G26-24 cells was found to be far lower than the amount of SFV-RNA synthesised in C1300 cells. SFV infected G26-24 cells were also found to exhibit a CPE at 8 hours post-infection, at which time SFV-infected C1300 cells appeared normal. These observations are consistent with the report by Atkins and Sheahan (Atkins and Sheahan, 1982) which concluded that G26-24 cells are sensitive to small amounts of SFV-RNA synthesis, whereas C1300 cells are able to tolerate extensive SFV-RNA synthesis without showing a CPE. The ability of the L10 strain of SFV to undergo protein synthesis within G26-24 and C1300 cells has also been reported by Atkins and Sheahan (Atkins and Sheahan, 1982). In the present study SFV antigen on the surface of infected G26-24 and C1300 cells was monitored, and it was found that at least 66% of SFV-infected G26-24 cells, and at least 61% of SFV infected C1300 cells display SFV antigen on the cell surface.



#### Chapter 4

##### Effect of IFN on SFV replication and MHC antigen display by brain cells

It is now well established that IFNs are able to induce the expression of MHC antigen on a variety of cell types in both the murine and human system (for review see chapter 1). In this chapter the effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  on class I and class II MHC antigen expression by astrocytes, G26-24 cells and C1300 cells is investigated. Furthermore, since both IFN- $\alpha\beta$  and IFN- $\gamma$  may be present within a virus-infected brain, the way in which natural IFN- $\alpha\beta$  and recombinant IFN- $\beta$  interact with recombinant IFN- $\gamma$  in the induction of class I and class II MHC antigen expression on astrocytes is also investigated.

In the previous chapter the ability of SFV to undergo replication in brain cells was investigated. IFNs by definition have an antiviral activity and in this chapter the relative sensitivities of astrocytes, G26-24 cells and C1300 cells to the antiviral effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  are assessed, via the inhibition of the incorporation of [ $^3$ H]uridine into SFV-RNA in infected cells. The effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  on SFV antigen expression by infected astrocytes, G26-24 cells and C1300 cells is also studied.

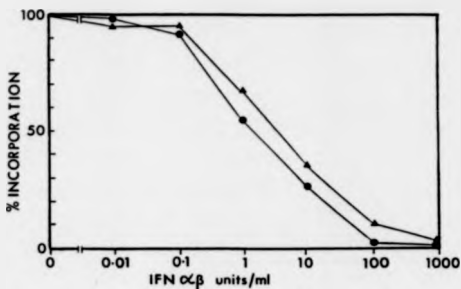
## Results

### Relative sensitivity of brain cells to IFN- $\alpha\beta$ and IFN- $\gamma$

In order to assess the relative sensitivity of astrocytes to IFN- $\alpha\beta$  and IFN- $\gamma$ , the incorporation of [ $^3$ H]uridine into IFN-treated SFV-infected astrocytes and L929 cells was determined. L929 cells are a fibroblast cell line derived from the C3H/He mouse known to be highly sensitive to both IFN- $\alpha\beta$  and IFN- $\gamma$  (Neager, 1987). Figures 4.1 and 4.2 show the incorporation of [ $^3$ H]uridine into IFN- $\alpha\beta$  and IFN- $\gamma$  treated SFV-infected astrocytes respectively, as compared with L929 cells. The data show that both cell types had about equal sensitivity to IFN- $\alpha\beta$  and IFN- $\gamma$ , with about 50% inhibition of incorporation of [ $^3$ H]uridine into viral RNA at 1 u/ml for both IFN- $\alpha\beta$  and IFN- $\gamma$ , and essentially no incorporation of [ $^3$ H]uridine above background levels (hence no detectable viral RNA synthesis) at 1000 u/ml IFN- $\alpha\beta$  and 100 u/ml IFN- $\gamma$ . The relative sensitivities of G26-24 cells and C1300 cells were also determined in the same manner. Figures 4.3 and 4.4 show the incorporation of [ $^3$ H]uridine into IFN- $\alpha\beta$  and IFN- $\gamma$  treated SFV-infected G26-24 cells respectively, as compared with L929 cells. The data show that both G26-24 cells and L929 cells had about equal sensitivity to IFN- $\alpha\beta$  and IFN- $\gamma$ , with about 50% inhibition of incorporation of [ $^3$ H]uridine into viral RNA at around 1 u/ml for both IFN- $\alpha\beta$  and IFN- $\gamma$ , and no incorporation of [ $^3$ H]uridine above background levels (hence no detectable viral RNA synthesis) at 300 u/ml IFN- $\alpha\beta$  and 500 u/ml IFN- $\gamma$ . Figure 4.5 shows the incorporation of [ $^3$ H]uridine into IFN- $\alpha\beta$  treated C1300 cells, as compared with L929 cells. The data show that C1300 cells were slightly less sensitive to IFN- $\alpha\beta$  than L929 cells, with only 23% inhibition of incorporation of [ $^3$ H]uridine at 1 u/ml IFN- $\alpha\beta$  for C1300 cells, as compared with 58% inhibition of incorporation in this experiment at 1 u/ml

Figure 4.1

Effect of IFN- $\alpha\beta$  on incorporation of [ $^3$ H]uridine into SFV-RNA by astrocytes and L929 cells



**Key**

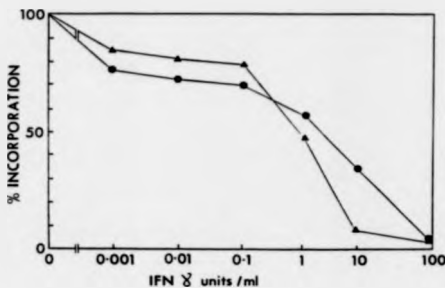
( $\Delta$ — $\Delta$ ) Astrocytes

( $\bullet$ — $\bullet$ ) L929 cells

- (1) Incorporation of [ $^3$ H]uridine is expressed as a percentage of that by SFV infected cells in the absence of IFN- $\alpha\beta$ , with the background of residual cellular incorporation (by uninfected cells; 3% or less that of infected cells) subtracted.
- (11) Assay protocol as described in methods section Q.

Figure 4.2

Effect of IFN- $\gamma$  on incorporation of [ $^3$ H]uridine into SFV-RNA by astrocytes and L929 cells



Key

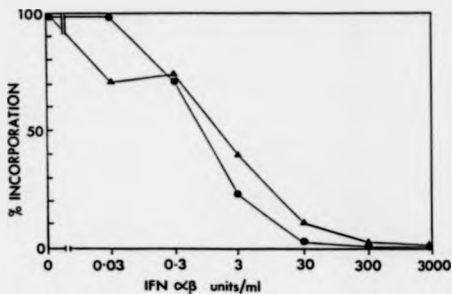
( $\Delta$ — $\Delta$ ) Astrocytes

( $\bullet$ — $\bullet$ ) L929 cells

- (i) Incorporation of [ $^3$ H]uridine is expressed as a percentage of that by SFV infected cells in the absence of IFN- $\gamma$ , with the background of residual cellular incorporation (by uninfected cells: 3% or less that of infected cells) subtracted.
- (ii) Assay protocol as described in methods section Q.

Figure 4.1

Effect of IFN- $\alpha\beta$  on incorporation of [ $^3$ H]uridine into SFV-RNA by G26-24 and L929 cells



**Key**

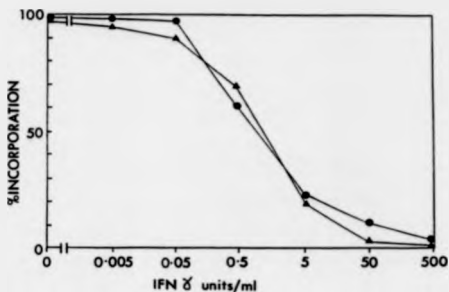
(●—●) G26-24 cells

(▲—▲) L929 cells

- (i) Incorporation of [ $^3$ H]uridine is expressed as a percentage of that by SFV infected cells in the absence of IFN- $\alpha\beta$ , with the background of residual cellular incorporation (by uninfected cells; 4% or less that of infected cells) subtracted.
- (ii) Assay protocol as described in methods section Q.

Figure 4.4

Effect of IFN- $\gamma$  on incorporation of [ $^3$ H]uridine into SFV-RNA by G26-24 and L929 cells



**Key**

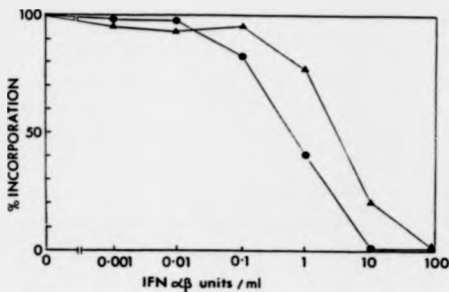
(●—●) G26-24 cells

(▲—▲) L929 cells

- (i) Incorporation of [ $^3$ H]uridine is expressed as a percentage of that by SFV infected cells in the absence of IFN- $\gamma$ , with the background of residual cellular incorporation (by uninfected cells; 32% or less that of infected cells) subtracted.
- (ii) Assay protocol as described in methods section Q.

Figure 4.5

Effect of IFN- $\alpha$  on incorporation of [ $^3$ H]uridine into SFV-RNA by C1300 and L929 cells



Key

( $\Delta$ — $\Delta$ ) C1300 cells

( $\bullet$ — $\bullet$ ) L929 cells

- (i) Incorporation of [ $^3$ H]uridine is expressed as a percentage of that by SFV infected cells in the absence of IFN- $\alpha$ , with the background of residual cellular incorporation (by uninfected cells; 8% or less that of infected cells) subtracted.
- (ii) Assay protocol as described in methods section Q.

IFN- $\alpha\beta$  for L929 cells. No incorporation of [ $^3$ H]uridine above background levels (hence no detectable viral RNA synthesis) was observed in this experiment at 10 u/ml IFN- $\alpha\beta$  for L929 cells, a concentration at which incorporation of [ $^3$ H]uridine into C1300 cells was only inhibited by 80%. No incorporation of [ $^3$ H]uridine above background levels (hence no detectable viral RNA synthesis) was observed at 100 u/ml IFN- $\alpha\beta$  for C1300 cells. Figure 4.6 shows the incorporation of [ $^3$ H]uridine into IFN- $\gamma$  treated SFV-infected C1300 cells, as compared with L929 cells. The data show that C1300 cells were far less sensitive to IFN- $\gamma$  than L929 cells, with about 20% inhibition of incorporation of [ $^3$ H]uridine at 1 u/ml IFN- $\gamma$  for C1300 cells, as compared with around 50% inhibition of incorporation in this experiment at 1 u/ml IFN- $\gamma$  for L929 cells. Essentially no incorporation of [ $^3$ H]uridine above background levels (hence no detectable viral RNA synthesis) was observed for L929 cells at 2000 u/ml IFN- $\gamma$ , whereas C1300 cells still incorporated 35% of [ $^3$ H]uridine at this concentration of IFN- $\gamma$ . Thus, viral RNA synthesis was still taking place in at least in some IFN- $\gamma$  treated SFV-infected C1300 cells at 2000 u/ml IFN- $\gamma$ . At the time at which these experiments were performed 2000 u/ml of IFN- $\gamma$  was the highest concentration of IFN- $\gamma$  which was available for use in these experiments. Whether concentrations of IFN- $\gamma$  greater than 2000 u/ml inhibit the incorporation of [ $^3$ H]uridine into viral RNA in C1300 cells further, remains to be determined.

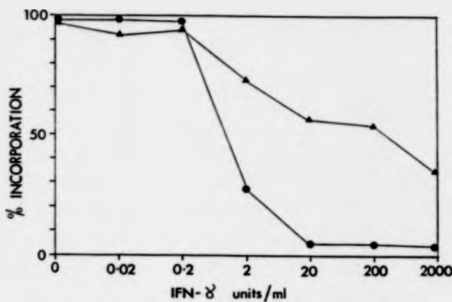
#### Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on SFV antigen expression by SFV-infected brain cells

In chapter 3, data were presented which showed that SFV-infected astrocytes, G26-24 cells and C1300 cells display SFV antigen on the cell surface. In this section the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on SFV



Figure 4.6

Effect of IFN- $\gamma$  on incorporation of [ $^3$ H]uridine into SFV-RNA by C1300 and L929 cells



**Key**

(▲—▲) C1300 cells

(●—●) L929 cells

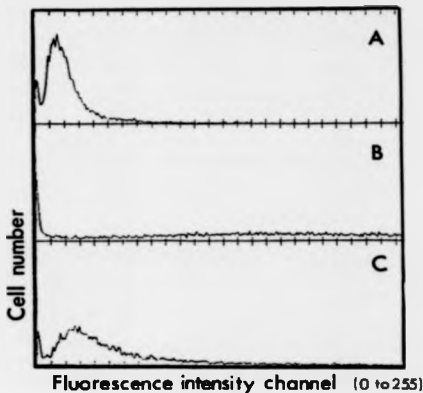
- (i) Incorporation of [ $^3$ H]uridine is expressed as a percentage of that by SFV infected cells in the absence of IFN- $\gamma$ , with the background of residual cellular incorporation (by uninfected cells; 6% or less that of infected cells) subtracted.
- (ii) Assay protocol as described in methods section Q.

antigen expression by the brain cells is investigated. The expression of SFV antigen was measured by indirect immunofluorescence staining with quantitation by flow cytometry.

Figure 4.7 shows the effect of IFN- $\alpha\beta$  on SFV antigen expression by astrocytes. It can be seen that SFV-infected astrocytes display high levels of SFV antigen on the cell surface, since the fluorescence of SFV-infected astrocytes was markedly higher than for uninfected astrocytes (fig 4.7a and b). Statistical analysis of the fluorescence data revealed a shift in mean fluorescence from 30 for uninfected astrocytes to 160 for SFV-infected astrocytes, and an increase in the percentage of cells collected in the maximum fluorescence intensity channel (channel 255) from 0% for uninfected astrocytes to 26% for SFV infected astrocytes. As shown in figure 4.7b and 4.7c, pretreatment of astrocytes with IFN- $\alpha\beta$ , prior to infection with SFV, markedly reduced the fluorescence of the astrocytes hence the display of SFV antigen on these cells. Statistical analysis of the fluorescence data revealed a shift in mean fluorescence from 160 for non-IFN treated SFV-infected astrocytes, to 66 for IFN- $\alpha\beta$  treated SFV-infected astrocytes. Pretreatment of astrocytes with IFN- $\alpha\beta$  did not totally abolish SFV antigen expression, since the mean fluorescence of IFN- $\alpha\beta$  treated SFV-infected astrocytes at 66 was still clearly elevated above that of uninfected cells at 30. Furthermore, statistical analysis of the fluorescence data showed that 4% of IFN- $\alpha\beta$  treated SFV-infected cells were still highly fluorescent and were collected in the maximum fluorescence intensity channel. Figure 4.8 shows the effect of IFN- $\gamma$  on SFV antigen expression by astrocytes. Again it can be seen that SFV-infected astrocytes display high levels of SFV antigen on the cell surface, since the fluorescence of SFV-infected astrocytes was markedly higher than for uninfected astrocytes (fig. 4.8a and b). Statistical analysis of the

Figure 4.7

Effect of IFN- $\alpha$  on expression of SFV antigen by SFV-infected astrocytes



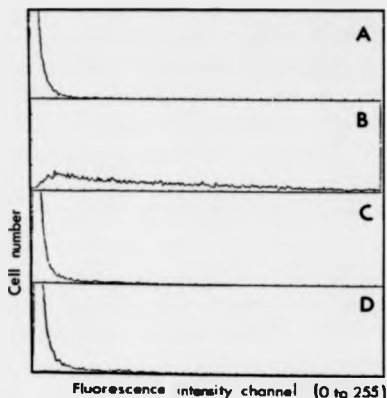
**Key**

- (A) non-IFN treated, uninfected
- (B) non-IFN treated, infected with SFV
- (C) treated with IFN- $\alpha$ , infected with SFV

- (i) Astrocytes were treated with 1000 u/ml IFN- $\alpha$ , or medium alone for 48 hours prior to infection with SFV, as described in methods section M. SFV antigens were stained by indirect immunofluorescence at 9 hours post-infection, as described in methods section M.
- (ii) SFV antigen expression was quantified using the FACS440 flow cytometer at the University of Birmingham, operated by Miss A Milner.
- (iii) 9728 cells analysed in each case.
- (iv) Data obtained by Mr M J Blackman (University of Warwick).

Figure 4.8

Effect of IFN- $\gamma$  on expression of SFV antigen by SFV-infected astrocytes



Key

- (A) non-IFN treated, uninfected
- (B) non-IFN treated, infected with SFV
- (C) treated with IFN- $\gamma$ , infected with SFV
- (D) non-IFN treated, treated with BPLSFV

- (i) Astrocytes were treated for 48 hours with 100 u/ml IFN- $\gamma$  or medium alone, prior to treatment with BPLSFV or infection with SFV, as described in methods section M. SFV antigens were then stained by indirect immunofluorescence at 7 hours post-infection, as described in methods section H.
- (ii) SFV antigen expression was quantified using the FACS440 flow cytometer at the University of Birmingham, operated by Miss A Milner.
- (iii) 9728 cells analysed in each case.
- (iv) BPLSFV;  $\beta$ -propiolactone inactivated preparation of SFV, see methods section B.

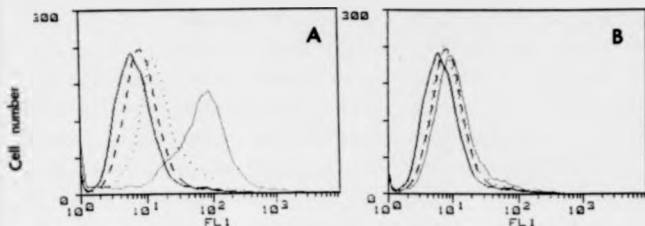
fluorescence data revealed a shift in mean fluorescence from 7 for uninfected astrocytes to 110 for SFV-infected astrocytes, and an increase in the percentage of cells collected above fluorescence intensity channel 13 from 10% for uninfected astrocytes to 96% for SFV-infected astrocytes. As shown in figure 4.8b and 4.8c, pretreatment of astrocytes with IFN- $\gamma$ , prior to infection with SFV, markedly reduced the fluorescence of the astrocytes hence the display of SFV antigen on these cells. Statistical analysis of the fluorescence data revealed a decrease in mean fluorescence from 110 for non-IFN treated SFV-infected astrocytes to 10 for IFN- $\gamma$  treated SFV-infected astrocytes, and a decrease in the percentage of cells collected above fluorescence intensity channel 13 from 96% for SFV-infected astrocytes to 14% for IFN- $\gamma$  treated SFV-infected astrocytes. The data obtained also suggest that pretreatment of astrocytes with IFN- $\gamma$  did not totally abolish SFV antigen expression, since the mean fluorescence of 10 for IFN- $\gamma$  treated SFV-infected astrocytes was slightly above that of 7 for uninfected astrocytes, and the percentage of cells collected above fluorescence intensity channel 13 at 14% for IFN- $\gamma$  treated SFV-infected astrocytes was slightly higher than 10% for uninfected cells. Thus, approximately 4% of IFN- $\gamma$  treated SFV-infected astrocytes may display very low levels of SFV antigen on the cell surface. To determine whether the input SFV antigen with which the cells were challenged could be detected on the surface of astrocytes, the cells were treated with an equivalent amount of a  $\beta$ -propiolactone inactivated preparation of SFV (BPLSFV). It can be seen that the fluorescence of BPLSFV treated astrocytes was essentially identical to that of uninfected cells (fig 4.8d). However, statistical analysis of the fluorescence data revealed that the mean fluorescence of 13 for BPLSFV-treated astrocytes was very slightly above that of 7 for uninfected astrocytes, and the percentage of cells collected above fluorescence intensity channel 13 at 20% for BPLSFV treated astrocytes was

higher than that of 10% for untreated astrocytes. Thus, the data suggest that at least 10% of BPLSFV-treated astrocytes may display very low levels of the input SFV antigen on the cell surface. The increased fluorescence of SFV-infected and BPLSFV-treated astrocytes, was specific for staining with anti-SFV serum, since the fluorescence of these cells stained with an irrelevant antibody was identical to that of untreated astrocytes stained with the same antibody (data not shown).

Figure 4.9 shows the effect of IFN- $\gamma$  on expression of SFV antigen by SFV-infected G26-24 cells. It can be seen that SFV-infected G26-24 cells display high levels of SFV antigen on the cell surface, since the fluorescence of SFV-infected G26-24 cells was markedly higher than for uninfected G26-24 cells (fig 4.9a). Statistical analysis of the fluorescence data revealed a shift in mean fluorescence from 23 for uninfected G26-24 cells to 141 for SFV-infected G26-24 cells, and an increase in the percentage of cells collected above fluorescence intensity channel 38 from 5% for uninfected G26-24 cells to 71% for SFV-infected G26-24 cells. As shown in fig 4.9a, pretreatment of G26-24 cells with IFN- $\gamma$ , prior to infection with SFV, markedly reduced the fluorescence of SFV-infected G26-24 cells hence the display of SFV antigen on the surface of these cells. Statistical analysis of the fluorescence data revealed a decrease in mean fluorescence from 141 for non-IFN treated SFV-infected G26-24 cells to 55 for IFN- $\gamma$  treated SFV-infected G26-24 cells, and a decrease in the percentage of cells collected above fluorescence intensity channel 38 from 71% for SFV-infected G26-24 cells to 16% for IFN- $\gamma$  treated SFV-infected G26-24 cells. Pretreatment of G26-24 cells with IFN- $\gamma$  did not totally abolish SFV antigen expression, since the mean fluorescence of 55 for IFN- $\gamma$  treated SFV-infected G26-24 cells was still clearly elevated above that of 27 for IFN- $\gamma$  treated uninfected G26-24 cells, and the

Figure 4.9

Effect of IFN- $\gamma$  on expression of SFV antigen by BPLSFV-treated and SFV-infected G26-24 cells



Key

- (A) solid line : non-IFN treated, uninfected  
 close dots : non-IFN treated, infected with SFV  
 spaced dots : treated with IFN- $\gamma$ , infected with SFV  
 dashed line : non-IFN treated, treated with BPLSFV
- (B) solid line : non-IFN treated, uninfected  
 spaced dots : treated with IFN- $\gamma$ , uninfected  
 close dots : treated with IFN- $\gamma$ , treated with BPLSFV  
 dashed line : non-IFN treated, treated with BPLSFV

FLI: Fluorescence intensity channel

\*: BPLSFV:  $\beta$ -propiolactone inactivated SFV (see methods section B)

- (1) G26-24 cells were treated with either 500 u/ml IFN- $\gamma$  or medium alone for 48 hours, prior to treatment with BPLSFV or infection with SFV, as described in methods section M. SFV antigens were then stained by indirect immunofluorescence at 8 hours post-infection, as described in methods section N.
- (11) 10 000 cells analysed in each case.

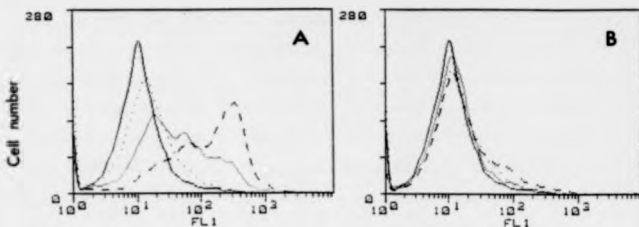
percentage of cells collected above fluorescence channel 38 at 160 for IFN- $\gamma$  treated SFV-infected G26-24 cells was above that of 60 for IFN- $\gamma$  treated uninfected G26-24 cells. Thus, at least 10% of IFN- $\gamma$  treated SFV-infected G26-24 cells display low levels of SFV antigen on the cell surface. To determine whether the input SFV antigen with which cells were challenged could be detected on the surface of G26-24 cells, the cells were treated with an equivalent amount of BPLSFV. It can be seen that the fluorescence of BPLSFV-treated G26-24 cells was slightly higher than that of uninfected G26-24 cells (fig 4.9b). Statistical analysis also revealed the mean fluorescence of 30 for BPLSFV-treated G26-24 cells to be slightly higher than of 23 for untreated G26-24 cells, indicating that at least some G26-24 cells may display low levels of the input SFV antigen on the cell surface. IFN- $\gamma$  treatment of G26-24 cells, prior to treatment with BPLSFV, slightly increased the fluorescence of the cells (fig 4.9b). However, this increase does not appear to be specific since IFN- $\gamma$  treatment also slightly increased the fluorescence of G26-24 cells stained with an irrelevant antibody (data not shown). The increased fluorescence of SFV-infected and BPLSFV-treated G26-24 cells, was specific for staining with anti-SFV serum, since the fluorescence of these cells stained with an irrelevant antibody was identical to that of untreated G26-24 cells stained with the same antibody (data not shown).

Figure 4.10 shows the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of SFV antigen by infected C1300 cells. It can be seen that SFV-infected C1300 cells display high levels of SFV antigen on the cell surface, since the fluorescence of SFV-infected C1300 cells was markedly higher than for uninfected C1300 cells (fig 4.10a). Statistical analysis of the fluorescence data revealed a shift in mean fluorescence from 33 for uninfected C1300 cells to 237 for SFV-infected C1300 cells, and an increase



Figure 4.10

Effect of IFN- $\alpha$  and IFN- $\gamma$  on expression of SFV antigen by BPLSFV-treated and SFV-infected C1300 cells



**Key**

- |     |             |   |  |
|-----|-------------|---|--|
| (A) | solid line  | : | non-IFN treated, uninfected                      |
|     | spaced dots | : | treated with IFN- $\alpha$ , infected with SFV   |
|     | close dots  | : | treated with IFN- $\gamma$ , infected with SFV   |
|     | dashed line | : | non-IFN treated, infected with SFV               |
| (B) | solid line  | : | non-IFN treated, uninfected                      |
|     | dashed line | : | non-IFN treated, treated with BPLSFV*            |
|     | close dots  | : | treated with IFN- $\alpha$ , treated with BPLSFV |
|     | spaced dots | : | treated with IFN- $\gamma$ , treated with BPLSFV |

FL1: Fluorescence intensity channel

\*; BPLSFV:  $\beta$ -propiolactone inactivated SFV (see methods section B)

- (1) C1300 cells were treated with either 1000 u/ml IFN- $\alpha$ , 1000 u/ml IFN- $\gamma$  or medium alone for 48 hours, prior to treatment with BPLSFV or infection with SFV, as described in methods section M. SFV antigens were then stained by indirect immunofluorescence at 9 hours post-infection, as described in methods section H.
- (11) 10 000 cells analysed in each case.

in the percentage of cells collected above fluorescence channel 63 from 5% for uninfected C1300 cells to 66% for SFV-infected C1300 cells. As shown in figure 4.10a, pretreatment of C1300 cells with IFN- $\alpha\beta$ , prior to infection with SFV, markedly reduced the fluorescence of SFV-infected C1300 cells hence the display of SFV antigen on the surface of these cells. Statistical analysis revealed a decrease in mean fluorescence from 239 for non-IFN treated SFV-infected C1300 cells to 64 for IFN- $\alpha\beta$  treated SFV-infected C1300 cells, and a decrease in the percentage of cells collected above fluorescence intensity channel 63 from 66% for SFV-infected C1300 cells to 9% for IFN- $\alpha\beta$  treated SFV-infected C1300 cells. Pretreatment of C1300 cells with IFN- $\alpha\beta$  did not totally abolish SFV antigen expression, since the mean fluorescence of 64 for IFN- $\alpha\beta$  treated SFV-infected C1300 cells was slightly higher than that of 33 for untreated C1300 cells, and the percentage of cells collected above fluorescence intensity channel 64 at 8% for IFN- $\alpha\beta$  treated SFV-infected C1300 cells was slightly above that of 5% for uninfected C1300 cells. Thus, at least 3% of IFN- $\alpha\beta$  treated SFV-infected C1300 cells display low levels of SFV antigen on the cell surface. As shown in figure 4.10a, pretreatment of C1300 cells with IFN- $\gamma$ , prior to infection with SFV, markedly reduced the fluorescence of a proportion of SFV-infected C1300 cells hence the display of SFV antigen on the surface of at least some of the cells. Statistical analysis of the fluorescence data revealed a decrease in mean fluorescence from 239 for non-IFN treated SFV-infected C1300 cells to 128 for IFN- $\gamma$  treated SFV-infected cells, and a decrease in the percentage of cells collected above fluorescence intensity channel 64 from 66% for SFV-infected C1300 cells to 34% for IFN- $\gamma$  treated SFV-infected C1300 cells. Pretreatment of C1300 cells with 1000 u/ml IFN- $\gamma$  quite clearly did not reduce SFV antigen display on all of the cells present, since the mean fluorescence of 128 for IFN- $\gamma$  treated SFV-infected C1300 cells was clearly elevated above that of 33 for untreated C1300

cells, and the percentage of cells collected above fluorescence channel 64 at 34% for IFN- $\gamma$  treated SFV-infected C1300 cells was substantially higher than that of 5% for untreated C1300 cells. Thus, at least 29% of IFN- $\gamma$  treated SFV-infected C1300 cells display SFV antigen on the cell surface, and observation of the fluorescence histogram shows that some of these cells display SFV antigen at a very high level (fig 4.10a). To determine whether the input SFV antigen with which cells were challenged could be detected on the surface of C1300 cells, the cells were treated with an equivalent amount of BPLSFV. It can be seen that the fluorescence of BPLSFV-treated C1300 cells was slightly higher than that of untreated C1300 cells (fig 4.10b). Statistical analysis of the data revealed the mean fluorescence of 45 for BPLSFV-treated C1300 cells to be slightly higher than that of 33 for untreated cells, and the percentage of BPLSFV-treated C1300 cells collected fluorescence channel 64 at 8% was higher than that of 5% for untreated C1300 cells. Thus, the data suggest that at least 3% of C1300 cells may display low levels of the input SFV antigen on the cell surface. As shown in figure 4.10b, treatment of C1300 cells with IFN- $\alpha\beta$  or IFN- $\gamma$ , prior to treatment with BPLSFV, slightly reduced the already low level of input SFV antigen displayed on the surface of these cells. The increased fluorescence of SFV-infected and BPLSFV-treated C1300 cells was specific for staining with anti-SFV serum, since the fluorescence of these cells stained with an irrelevant antibody was essentially identical to that of untreated C1300 cells stained with the same antibody (data not shown).

#### Effect of IFN- $\alpha\beta$ and IFN- $\gamma$ treatment on expression of class I and class II MHC antigen by brain cells

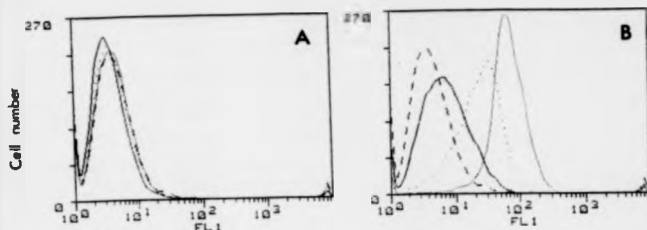
In this section the effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  treatment on expression of class I and class II MHC antigen by astrocytes,

G26-24 cells and C1300 cells is investigated. The expression of both major class I MHC determinants, H-2D and H-2K, and the class II MHC determinant H-2A, was assessed by indirect immunofluorescence staining with quantitation by flow cytometry. An indirect measure of functional class I MHC antigen expression was also obtained by measuring the susceptibility of the cells to lysis by alloreactive cytotoxic-T-lymphocytes (CTL) since these recognise class I MHC determinants alone (Kuppers *et al.*, 1981). In some cases a measure of functional class II MHC expression was obtained by assaying the ability of cells to stimulate an allogeneic mixed lymphocyte reaction (Takiguchi *et al.*, 1986).

Figures 4.11 and 4.12 show the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of class I MHC H-2D<sup>k</sup> antigen by astrocytes. As shown in figure 4.11a and 4.12a, at least some untreated astrocytes expressed very low levels of H-2D<sup>k</sup> antigen since the fluorescence of anti-H-2D<sup>k</sup> stained cells was slightly higher than for the same cells stained with an irrelevant antibody anti-H-2D<sup>d</sup> (background fluorescence). Statistical analysis of the fluorescence data showed that at least 1% of untreated astrocytes displayed H-2D<sup>k</sup> antigen on the cell surface. IFN- $\alpha\beta$  treatment markedly increased H-2D<sup>k</sup> antigen expression on the astrocytes (fig 4.11a and b). The augmentation of H-2D<sup>k</sup> antigen was first observed at a concentration of 10 u/ml IFN- $\alpha\beta$  on at least 19% of the astrocytes, and increased thereafter in a dose-dependant manner. Statistical analysis showed that at least 91% of astrocytes expressed H-2D<sup>k</sup> antigen when treated with 1000 u/ml IFN- $\alpha\beta$ . IFN- $\gamma$  treatment also markedly increased H-2D<sup>k</sup> antigen expression on the astrocytes (fig 4.12a and b). The augmentation of H-2D<sup>k</sup> antigen by IFN- $\gamma$  was first observed at a concentration of 0.1 u/ml on at least 37% of the astrocytes, and increased thereafter in a dose-dependant manner. Statistical analysis showed that at least 94% of astrocytes expressed H-2D<sup>k</sup>

**Figure 4.11**

**Effect of IFN- $\alpha\beta$  on expression of class I MHC H-2D<sup>k</sup> antigen by astrocytes**



**Key**

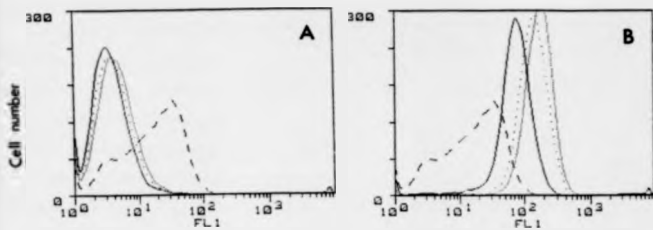
- (A) solid line : background fluorescence  
 spaced dots : untreated  
 close dots : 0.1 u/ml IFN- $\alpha\beta$   
 dashed line : 1 u/ml IFN- $\alpha\beta$
- (B) dashed line : 1 u/ml IFN- $\alpha\beta$   
 solid line : 10 u/ml IFN- $\alpha\beta$   
 spaced dots : 100 u/ml IFN- $\alpha\beta$   
 close dots : 1000 u/ml IFN- $\alpha\beta$

FL1; Fluorescence intensity channel

- (i) Astrocytes were treated for 48 hours with the appropriate concentration of IFN- $\alpha\beta$  prior to staining H-2D<sup>k</sup> antigens by indirect immunofluorescence and quantitation by flow cytometry as described in methods section H.
- (ii) 10 000 cells analysed in each case.

Figure 4.12

Effect of IFN- $\gamma$  on expression of class I MHC H-2b<sup>k</sup> antigen by astrocytes



Key

- (A) solid line : background fluorescence  
 spaced dots : untreated  
 close dots : 0.01 u/ml IFN- $\gamma$   
 dashed line : 0.1 u/ml IFN- $\gamma$
- (B) dashed line : 0.1 u/ml IFN- $\gamma$   
 solid line : 1 u/ml IFN- $\gamma$   
 spaced dots : 10 u/ml IFN- $\gamma$   
 close dots : 100 u/ml IFN- $\gamma$

FLI; Fluorescence intensity channel

- (i) Astrocytes were treated for 48 hours with the appropriate concentration of IFN- $\gamma$  prior to staining M-2b<sup>k</sup> antigens by indirect immunofluorescence and quantitation by flow cytometry as described in methods section H.
- (ii) 10 000 cells analysed in each case.

antigen when treated with 100 u/ml IFN- $\gamma$ . Figures 4.13 and 4.14 show the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of class I MHC H-2K<sup>k</sup> antigen by astrocytes. As shown in figure 4.13a and 4.14a, some untreated astrocytes displayed moderate levels of H-2K<sup>k</sup> antigen on the cell surface, since the fluorescence of anti-H-2K<sup>k</sup> stained cells was clearly elevated above that of the same cells stained with an irrelevant antibody anti-H-2D<sup>d</sup> (background fluorescence). Statistical analysis of the fluorescence data showed that at least 66% of untreated astrocytes displayed H-2K<sup>k</sup> antigen in the experiment shown in figure 4.13a, and at least 26% in the experiment shown in figure 4.14a. IFN- $\alpha\beta$  treatment markedly increased H-2K<sup>k</sup> antigen expression on the astrocytes (fig 4.13a and b). The augmentation of H-2K<sup>k</sup> antigen was first observed at a concentration of 10 u/ml IFN- $\alpha\beta$  on at least 7% of the astrocytes, and increased thereafter in a dose-dependant manner. Statistical analysis showed that at least 92% of astrocytes expressed H-2K<sup>k</sup> antigen when treated with 1000 u/ml IFN- $\alpha\beta$ . IFN- $\gamma$  treatment also markedly increased H-2K<sup>k</sup> antigen expression on the astrocytes (fig 4.14a and b). The augmentation of H-2K<sup>k</sup> antigen by IFN- $\gamma$  was first observed at a concentration of 0.01 u/ml on at least 11% of the astrocytes, and increased thereafter in a dose-dependant manner upto 10 u/ml. The fluorescence of astrocytes treated with 100 u/ml IFN- $\gamma$  was essentially identical to that for astrocytes treated with 10 u/ml IFN- $\gamma$ , suggesting that the cells were expressing maximum levels of H-2K<sup>k</sup> antigen. Statistical analysis showed that at least 92% of astrocytes expressed H-2K<sup>k</sup> antigen when treated with 100 u/ml IFN- $\gamma$ . The increased fluorescence of IFN- $\alpha\beta$  and IFN- $\gamma$  treated astrocytes shown in figures 4.11, 4.12, 4.13 and 4.14 was specific for staining H-2D<sup>k</sup> and H-2K<sup>k</sup> antigen, since the fluorescence of IFN-treated astrocytes stained with an irrelevant antibody was identical to that of non-IFN treated astrocytes stained with the same antibody (data not shown). Similarly, the increased fluorescence of IFN- $\alpha\beta$  and IFN- $\gamma$  treated cells

Figure 4.13

Effect of IFN- $\alpha$  on expression of class I H-2K<sup>b</sup> antigen by astrocytes



**Key**

- (A) solid line : background fluorescence  
 spaced dots : untreated  
 close dots : 0.1 u/ml IFN- $\alpha$   
 dashed line : 1 u/ml IFN- $\alpha$
- (B) dashed line : 1 u/ml IFN- $\alpha$   
 solid line : 10 u/ml IFN- $\alpha$   
 spaced dots : 100 u/ml IFN- $\alpha$   
 close dots : 1000 u/ml IFN- $\alpha$

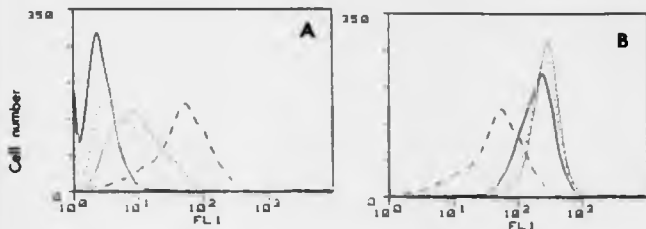
FL1: Fluorescence intensity channel

- (i) Astrocytes were treated for 48 hours with the appropriate concentration of IFN- $\alpha$  prior to staining H-2K<sup>b</sup> antigens by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells analysed in each case.



**Figure 4.14**

**Effect of IFN- $\gamma$  on expression of class I MHC H-2K<sup>b</sup> antigen by astrocytes**



**Key**

- (A) solid line : background fluorescence  
 spaced dots : untreated  
 close dots : 0.01 u/ml IFN- $\gamma$   
 dashed line : 0.1 u/ml IFN- $\gamma$
- (B) dashed line : 0.1 u/ml IFN- $\gamma$   
 solid line : 1 u/ml IFN- $\gamma$   
 spaced dots : 10 u/ml IFN- $\gamma$   
 close dots : 100 u/ml IFN- $\gamma$

FLI: Fluorescence intensity channel

- (i) Astrocytes were treated for 48 hours with the appropriate concentration of IFN prior to staining H-2K<sup>b</sup> antigens by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells analysed in each case.

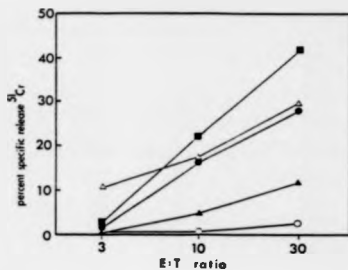
shown in the subsequent figures 4.16 to 4.33 was specific for staining the antigen under investigation, since unless stated otherwise the fluorescence of IFN-treated cells stained with an irrelevant antibody was always identical to that of non-IFN treated cells stained with the same antibody (data not shown).

Figure 4.15 shows the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on the susceptibility of astrocytes to lysis by alloreactive CTL. It can be seen that untreated astrocytes were slightly more susceptible to alloreactive CTL lysis than untreated control cells of the wrong haplotype (P815). Although it is possible that astrocytes and P815 cells differ in the background level of susceptibility to alloreactive CTL lysis, this observation does at least indicate that some astrocytes may express a sufficient level of functional class I MHC antigen for T-cell recognition to occur. IFN- $\alpha\beta$  and IFN- $\gamma$  treatment of the astrocytes markedly increased the susceptibility to alloreactive CTL lysis, indicating that both IFN types increased the ability of astrocytes to participate in class I MHC restricted T-cell mediated immune reactions. This increased ability may be due to an increased level of functional class I MHC antigen and/or adhesion molecule display on the cells. The killing by alloreactive CTL was H-2<sup>k</sup> specific since RDM4 cells (H-2<sup>k</sup>) were lysed whereas P815 cells (H-2<sup>d</sup>) were not.

Figures 4.16 and 4.17 show the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of class II MHC H-2A<sup>k</sup> antigen by astrocytes. As shown in figure 4.16a and 4.17a, untreated astrocytes displayed no detectable H-2A<sup>k</sup> antigen since the fluorescence of anti-H-2A<sup>k</sup> stained cells was identical to that of the same cells stained with an irrelevant antibody anti-H-2D<sup>d</sup> (background fluorescence). Statistical analysis of the fluorescence data revealed that

Figure 4.13

Effect of IFN- $\alpha$  and IFN- $\gamma$  treatment on susceptibility of astrocytes to lysis by alloreactive CTL



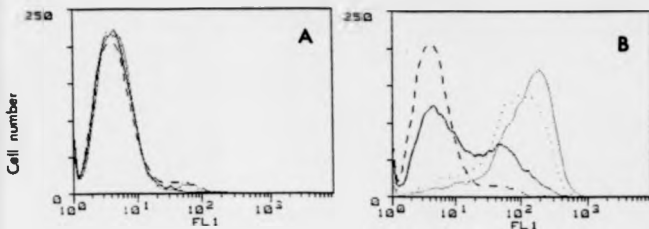
Key

- (▲—▲) Astrocytes (H-2<sup>k</sup>), untreated
- (●—●) Astrocytes (H-2<sup>k</sup>), treated with 100 units/ml IFN- $\gamma$
- (■—■) Astrocytes (H-2<sup>k</sup>), treated with 1000 units/ml IFN- $\alpha$
- (○—○) P815 cells (H-2<sup>d</sup>), untreated
- (△—△) RDM4 cells (H-2<sup>k</sup>), untreated

- (i) Cells were treated with IFN- $\alpha$ , IFN- $\gamma$  or medium alone for 48 hours. The cells were then harvested and susceptibility to alloreactive CTL lysis measured in a 3 hour chromium release assay, as described in methods section M.
- (ii) Effector alloreactive CTL were Balb/c-anti-C3H/He (H-2<sup>d</sup>-anti-H-2<sup>k</sup>) alloline.
- (iii) E:T ratio; Effector to Target ratio.

Figure 4.16

Effect of IFN- $\gamma$  on expression of class II MHC H-2A<sup>k</sup> antigen by astrocytes



Key

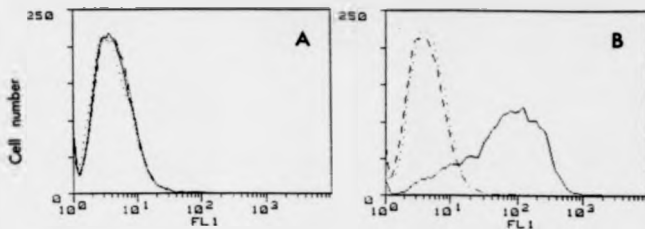
- (A) solid line : background fluorescence  
 spaced dots : untreated  
 close dots : 0.01 u/ml IFN- $\gamma$   
 dashed line : 0.1 u/ml IFN- $\gamma$
- (B) dashed line : 0.1 u/ml IFN- $\gamma$   
 solid line : 1 u/ml IFN- $\gamma$   
 spaced dots : 10 u/ml IFN- $\gamma$   
 close dots : 100 u/ml IFN- $\gamma$

FL1; Fluorescence intensity channel

- (i) Astrocytes were treated for 48 hours with the appropriate concentration of IFN- $\gamma$ , prior to staining H-2A<sup>k</sup> antigens by indirect immunofluorescence as described in methods section N.
- (ii) 10 000 cells analysed in each case.

Figure 4.17

Effect of IFN- $\alpha$  on expression of class II MHC H-2A<sup>k</sup> antigen by astrocytes



Key

- (A) solid line : background fluorescence  
 spaced dots : untreated  
 close dots : 10 u/ml IFN- $\alpha$   
 dashed line : 100 u/ml IFN- $\alpha$
- (B) dashed line : 100 u/ml IFN- $\alpha$   
 spaced dots : 1000 u/ml IFN- $\alpha$   
 solid line : 10 u/ml IFN- $\gamma$

FL1: Fluorescence intensity channel

- (i) Astrocytes were treated for 48 hours with the appropriate concentration of IFN- $\alpha$  or IFN- $\gamma$ , prior to staining H-2A<sup>k</sup> antigens by indirect immunofluorescence as described in methods section M.
- (ii) 10 000 cells analysed in each case.

at least 1% of untreated astrocytes display low levels of H-2A<sup>k</sup> antigen in the experiment shown in figure 4.16a, and 0% in the experiment shown in figure 4.17a. IFN- $\gamma$  treatment markedly increased H-2A<sup>k</sup> antigen expression on the astrocytes (fig 4.16a and b). The augmentation of H-2A<sup>k</sup> antigen was first clearly observed at a concentration of 1 u/ml IFN- $\gamma$  on at least 34% of the astrocytes, and increased thereafter in a dose-dependant manner. Statistical analysis showed that at least 86% of astrocytes expressed H-2A<sup>k</sup> antigen when treated with 100 u/ml IFN- $\gamma$ . In contrast to IFN- $\gamma$ , concentrations of IFN- $\alpha\beta$  between 1 and 1000 u/ml had no effect upon H-2A<sup>k</sup> antigen expression on the astrocytes, since the fluorescence of IFN- $\alpha\beta$  treated anti-H-2A<sup>k</sup> stained cells was identical to that of untreated cells stained with the same antibody (fig 4.17). In a separate experiment 10 000 u/ml IFN- $\alpha\beta$  was also found to have no effect on H-2A<sup>k</sup> antigen expression (data not shown).

A measure of functional class II MHC antigen expression on astrocytes was also obtained by assaying the ability of the cells to stimulate an allogeneic mixed lymphocyte reaction (MLR). As shown in table 4.1 treatment of astrocytes with 1000 u/ml IFN- $\alpha\beta$  did not increase the ability to stimulate an allogeneic MLR, since levels of [<sup>3</sup>H]thymidine incorporated by the responder lymphocytes stimulated by untreated and IFN- $\alpha\beta$  treated astrocytes were essentially identical. In contrast, astrocytes treated with 100 u/ml IFN- $\gamma$  clearly stimulated an allogeneic MLR with levels of [<sup>3</sup>H]thymidine incorporated by the responders rising from 8920 to 29971 c.p.m. (table 4.1). It is not possible to determine whether untreated astrocytes display any functional class II MHC antigen from the data obtained.

Figures 4.18 and 4.19 show the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of

Table 4.1

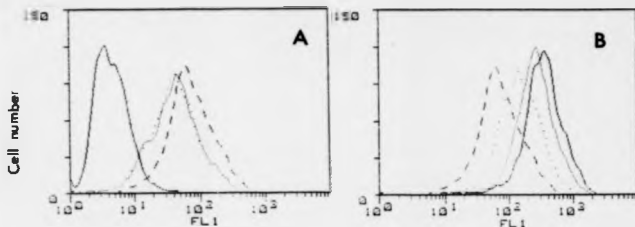
Effect of IFN- $\alpha$  and IFN- $\gamma$  treatment on ability of astrocytes to stimulate an allogeneic mixed lymphocyte reaction

Stimulator astrocyte	Responder:Stimulator Ratio		Stimulator
	10:1	3:1	Alone
Untreated	8920 $\pm$ 1295	7925 $\pm$ 1211	4094 $\pm$ 1675
IFN- $\alpha$ treated	8815 $\pm$ 1388	8959 $\pm$ 1933	2912 $\pm$ 1373
IFN- $\gamma$ treated	29971 $\pm$ 2074	26992 $\pm$ 2858	2674 $\pm$ 300

- (i) Astrocytes were treated for 48 hours with either 100 u/ml IFN- $\gamma$ , 1000 u/ml IFN- $\alpha$  or medium alone. The cells were then harvested and the ability to stimulate an allogeneic mixed lymphocyte reaction assessed as described in methods section O.
- (ii) Responders were Balb/c-anti-C3H/He (H-2<sup>d</sup>-anti-H-2<sup>k</sup>) allolines.
- (iii) Results were the mean of quadruplicate cultures and are expressed as Mean CPM  $\pm$  S.D. of [<sup>3</sup>H]thymidine incorporated.

Figure 4.18

Effect of IFN- $\alpha\beta$  on expression of class I MHC H-2D<sup>b</sup> antigen by G26-24 cells



Key

- (A) solid line : background fluorescence  
 spaced dots : untreated  
 close dots : 0.1 u/ml IFN- $\alpha\beta$   
 dashed line : 1 u/ml IFN- $\alpha\beta$
- (B) dashed line : 1 u/ml IFN- $\alpha\beta$   
 spaced dots : 10 u/ml IFN- $\alpha\beta$   
 close dots : 100 u/ml IFN- $\alpha\beta$   
 solid line : 1000 u/ml IFN- $\alpha\beta$

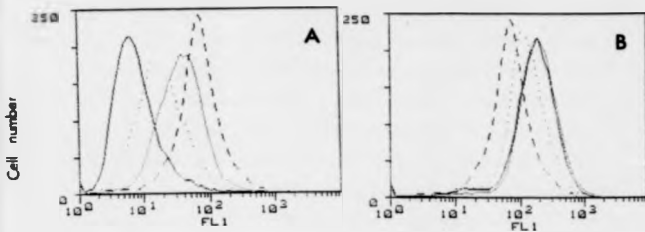
FL1: Fluorescence intensity channel

- (i) G26-24 cells were treated for 48 hours with the appropriate concentration of IFN- $\alpha\beta$  prior to staining H-2D<sup>b</sup> antigens by indirect immunofluorescence as described in methods section H.
- (ii) 5000 cells analysed in each case.



Figure 4.19

Effect of IFN- $\gamma$  on expression of class I HMG M-2D<sup>b</sup> antigen by G26-24 cells



**Key**

- (A) solid line : background fluorescence  
spaced dots : untreated  
close dots : 0.05 u/ml IFN- $\gamma$   
dashed line : 0.5 u/ml IFN- $\gamma$
- (B) dashed line : 0.5 u/ml IFN- $\gamma$   
spaced dots : 5 u/ml IFN- $\gamma$   
close dots : 50 u/ml IFN- $\gamma$   
solid line : 500 u/ml IFN- $\gamma$

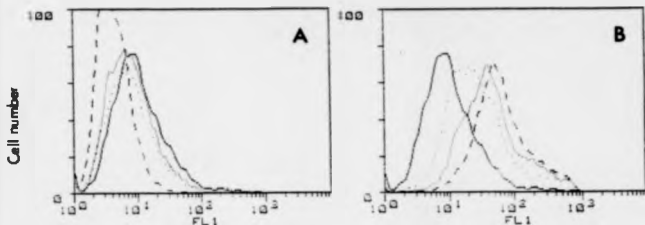
FL1; Fluorescence intensity channel

- (i) G26-24 cells were treated for 48 hours with the appropriate concentration of IFN- $\gamma$  prior to staining M-2D<sup>b</sup> antigens by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells analysed in each case.

class I MHC H-2D<sup>b</sup> antigen by G26-24 cells. As shown in figure 4.18a and 4.19a, some untreated G26-24 cells displayed moderate levels of H-2D<sup>b</sup> antigen on the cell surface, since the fluorescence of anti-H-2D<sup>b</sup> stained cells was clearly elevated above that of the same cells stained with an irrelevant antibody anti-M-2K<sup>d</sup> (background fluorescence). Statistical analysis of the fluorescence data showed that at least 84% of untreated G26-24 cells displayed H-2D<sup>b</sup> antigen in the experiment shown in figure 4.18a, and at least 12% in the experiment shown in figure 4.19a. IFN- $\alpha\beta$  treatment markedly increased H-2D<sup>b</sup> antigen expression on G26-24 cells (fig 4.18a and b). The augmentation of H-2D<sup>b</sup> antigen expression was first observed at a concentration of 1 u/ml IFN- $\alpha\beta$  on at least 10% of G26-24 cells, and increased thereafter in a dose-dependant manner. Statistical analysis showed that at least 92% of G26-24 cells expressed H-2D<sup>b</sup> antigen when treated with 1000 u/ml IFN- $\alpha\beta$ . IFN- $\gamma$  treatment also markedly increased H-2D<sup>b</sup> antigen expression on G26-24 cells (fig 4.19a and b). The augmentation of H-2D<sup>b</sup> antigen by IFN- $\gamma$  was first observed at a concentration of 0.05 u/ml IFN- $\gamma$  on at least 28% of G26-24 cells, and increased thereafter in a dose-dependant manner upto 50 u/ml. The fluorescence of G26-24 cells treated with 500 u/ml IFN- $\gamma$  was essentially identical to that of G26-24 cells treated with 50 u/ml IFN- $\gamma$ , suggesting that the cells were expressing maximum levels of H-2D<sup>b</sup> antigen. Statistical analysis showed that at least 87% of G26-24 cells expressed H-2D<sup>b</sup> antigen when treated with 500 u/ml IFN- $\gamma$ . Figures 4.20 and 4.21 show the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of class I MHC H-2K<sup>b</sup> antigen by G26-24 cells. As shown in figure 4.20a and 4.21a, some untreated G26-24 cells displayed moderate levels of H-2K<sup>b</sup> antigen on the cell surface, since the fluorescence of anti-M-2K<sup>b</sup> stained cells was clearly elevated above that of the same cells stained with an irrelevant antibody anti-M-2K<sup>d</sup> (background fluorescence). Statistical analysis of the fluorescence data

Figure 4.20

Effect of IFN- $\alpha$  on expression of class I H-2K<sup>b</sup> antigen by G26-24 cells



**Key**

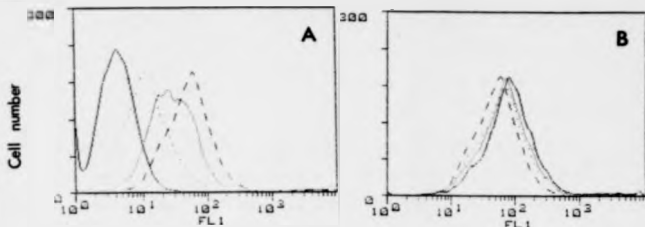
- (A) dashed line : background fluorescence  
 close dots : untreated  
 spaced dots : 0.1 u/ml IFN- $\alpha$   
 solid line : 1 u/ml IFN- $\alpha$
- (B) solid line : 1 u/ml IFN- $\alpha$   
 spaced dots : 10 u/ml IFN- $\alpha$   
 close dots : 100 u/ml IFN- $\alpha$   
 dashed line : 1000 u/ml IFN- $\alpha$

FL1; Fluorescence intensity channel

- (1) G26-24 cells were treated for 48 hours with the appropriate concentration of IFN- $\alpha$  prior to staining H-2K<sup>b</sup> antigens by indirect immunofluorescence as described in methods section A.
- (ii) 3600 to 4500 cells analyzed in each case.

Figure 4.21

Effect of IFN- $\gamma$  on expression of class I MHC H2-K<sup>b</sup> antigen by G26-24 cells



**Key**

- (A) solid line : background fluorescence  
spaced dots : untreated  
close dots : 0.05 u/ml IFN- $\gamma$   
dashed line : 0.5 u/ml IFN- $\gamma$
- (B) dashed line : 0.5 u/ml IFN- $\gamma$   
spaced dots : 5 u/ml IFN- $\gamma$   
close dots : 50 u/ml IFN- $\gamma$   
solid line : 500 u/ml IFN- $\gamma$

FLI: Fluorescence intensity distribution

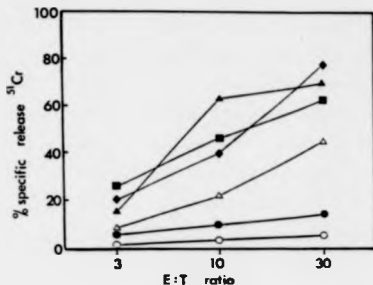
- (i) G26-24 cells were treated for 48 hours with the appropriate concentration of IFN- $\gamma$  prior to staining H2-K<sup>b</sup> antigens by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells analysed in each case.

showed that at least 15% of untreated G26-24 cells displayed H-2K<sup>b</sup> antigen in the experiment shown in figure 4.20a, and at least 42% in the experiment shown in figure 4.21a. IFN- $\alpha\beta$  treatment markedly increased H-2K<sup>b</sup> antigen expression on the G26-24 cells (fig 4.20a and b). The augmentation of H-2K<sup>b</sup> antigen was first observed at a concentration of 1 u/ml IFN- $\alpha\beta$  on at least 3% of the G26-24 cells, and increased thereafter in a dose-dependant manner. Statistical analysis showed that at least 65% of G26-24 cells expressed H-2K<sup>b</sup> antigen when treated with 1000 u/ml IFN- $\alpha\beta$ . IFN- $\gamma$  treatment also increased H-2K<sup>b</sup> antigen expression on the G26-24 cells (fig 4.21a and b). The augmentation of H-2K<sup>b</sup> antigen by IFN- $\gamma$  was first observed at a concentration of 0.05 u/ml on at least 15% of the G26-24 cells, and increased thereafter in a dose-dependant manner. Statistical analysis showed that 92% of G26-24 cells expressed H-2K<sup>b</sup> antigen when treated with 500 u/ml IFN- $\gamma$ .

Figure 4.22 shows the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on the susceptibility of G26-24 cells to lysis by alloreactive CTL. It can be seen that untreated G26-24 cells were clearly more susceptible to alloreactive CTL lysis than untreated control cells of the wrong haplotype (L929). Although the possibility exists that G26-24 cells and L929 cells differ in the background level of susceptibility to alloreactive CTL lysis, this observation does at least indicate that some G26-24 cells may express a sufficient level of functional class I MHC antigen for T-cell recognition to occur. IFN- $\alpha\beta$  and IFN- $\gamma$  treatment of G26-24 cells markedly increased the susceptibility to alloreactive CTL lysis, indicating that both IFN types increased the ability of G26-24 cells to participate in class I MHC restricted T-cell mediated immune reactions. This increased ability may be due to an increased level of functional class I MHC antigen and/or adhesion molecule display on the cells. The killing by alloreactive CTL was H-2<sup>b</sup>

Figure 4.22

Effect of IFN- $\alpha$  and IFN- $\gamma$  treatment on susceptibility of G26-24 cells to lysis by alloreactive CTL



**Key**

- ( $\Delta$ — $\Delta$ ) G26-24 cells (H-2<sup>b</sup>), untreated
- ( $\blacktriangle$ — $\blacktriangle$ ) G26-24 cells (H-2<sup>b</sup>), treated with 500 units/ml IFN- $\gamma$
- ( $\blacklozenge$ — $\blacklozenge$ ) G26-24 cells (H-2<sup>b</sup>), treated with 1000 units/ml IFN- $\alpha$
- ( $\blacksquare$ — $\blacksquare$ ) EIA cells (H-2<sup>b</sup>), untreated
- ( $\bullet$ — $\bullet$ ) L929 cells (H-2<sup>k</sup>), untreated
- ( $\circ$ — $\circ$ ) Yac-1 cells (H-2<sup>a</sup>), untreated

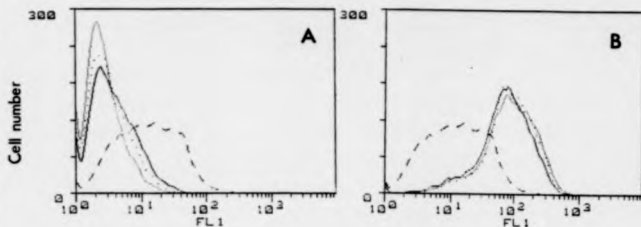
- (i) Cells were treated with IFN- $\alpha$ , IFN- $\gamma$  or medium alone for 48 hours. The cells were then harvested and susceptibility to alloreactive CTL lysis measured in a 4.5 hr chromium release assay, as described in methods section M.
- (ii) Effector alloreactive CTL were Balb/c-anti-C57 BL/6 (H-2<sup>d</sup>-anti-H-2<sup>b</sup>) allolines.
- (iii) E:T; Effector to Target ratio.

specific since ELA cells ( $H-2^m$ ) were lysed whereas L929 cells ( $H-2^k$ ) were not. Yac-1 cells were not lysed, showing that there was no significant natural killer cell activity in the effector population.

Figures 4.23 and 4.24 show the effect of  $IFN-\alpha\beta$  and  $IFN-\gamma$  on expression of class II MHC  $H-2A^b$  antigen by G26-24 cells. As shown in figure 4.23a and 4.24a, untreated G26-24 cells displayed no detectable  $H-2A^b$  antigen since the fluorescence of anti- $H-2A^b$  stained cells was identical to that of the same cells stained with an irrelevant antibody anti- $H-2K^d$  (background fluorescence). Statistical analysis also showed that 0% of untreated G26-24 cells displayed  $H-2A^b$  antigen on the cell surface.  $IFN-\gamma$  treatment markedly increased  $H-2A^b$  antigen expression on G26-24 cells (fig 4.23a and b). The augmentation of  $H-2A^b$  antigen was first observed at a concentration of 0.5 u/ml  $IFN-\gamma$  on at least 41% of G26-24 cells, and increased thereafter in a dose-dependant manner upto 50 u/ml  $IFN-\gamma$ . The fluorescence of G26-24 cells treated with 500 u/ml  $IFN-\gamma$  was identical to that of G26-24 cells treated with 50 u/ml  $IFN-\gamma$ , suggesting that the cells were expressing maximum levels of  $H-2A^b$  antigen. Statistical analysis showed that at least 87% of G26-24 cells expressed  $H-2A^b$  antigen when treated with 500 u/ml  $IFN-\gamma$ . In contrast to  $IFN-\gamma$ , concentrations of  $IFN-\alpha\beta$  between 1 and 100 u/ml had no effect upon  $H-2A^b$  antigen expression on G26-24 cells, since the fluorescence of  $IFN-\alpha\beta$  treated anti- $H-2A^b$  stained cells was identical to that of untreated cells stained with the same antibody (fig 4.24a and b). As shown in figure 4.24b, the fluorescence of anti- $H-2A^b$  stained G26-24 cells treated with 1000 u/ml  $IFN-\alpha\beta$  was slightly higher than for untreated G26-24 cells. This increase in fluorescence was not specific for staining  $H-2A^b$  antigen since this concentration of  $IFN-\alpha\beta$  also slightly increased the fluorescence of G26-24 cells stained with an irrelevant antibody (data not shown).

Figure 4.23

Effect of IFN- $\gamma$  on expression of class II MHC H-2A<sup>b</sup> antigen by G26-24 cells



Key

- (A) solid line : background fluorescence  
spaced dots : untreated  
close dots : 0.05 u/ml IFN- $\gamma$   
dashed line : 0.5 u/ml IFN- $\gamma$
- (B) dashed line : 0.5 u/ml IFN- $\gamma$   
solid line : 5 u/ml IFN- $\gamma$   
spaced dots : 50 u/ml IFN- $\gamma$   
close dots : 500 u/ml IFN- $\gamma$

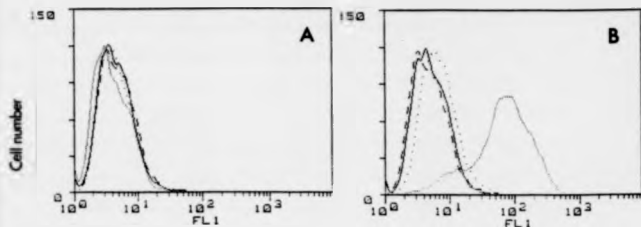
FL1; Fluorescence intensity channel

- (i) G26-24 cells were treated for 48 hours with the appropriate concentration of IFN- $\gamma$  prior to staining H-2A<sup>b</sup> antigens by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells analysed in each case.



Figure 4.24

Effect of IFN- $\alpha\beta$  on expression of class II H-2A<sup>b</sup> antigen by G26-24 cells



Key

- (A) solid line : background fluorescence  
spaced dots : untreated  
close dots : 1 u/ml IFN- $\alpha\beta$   
dashed line : 10 u/ml IFN- $\alpha\beta$
- (B) dashed line : 10 u/ml IFN- $\alpha\beta$   
solid line : 100 u/ml IFN- $\alpha\beta$   
spaced dots : 1000 u/ml IFN- $\alpha\beta$   
close dots : 500 u/ml IFN- $\gamma$

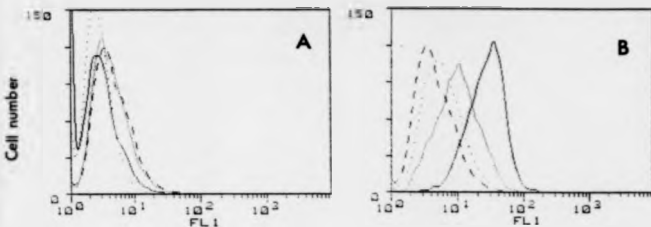
FL1; Fluorescence intensity channel

- (i) G26-24 cells were treated for 48 hours with the appropriate concentration of IFN prior to staining H-2A<sup>b</sup> antigens by indirect immunofluorescence as described in methods section H.
- (ii) 5000 cells analysed in each case.

Figures 4.25 and 4.26 show the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of class I MHC H-2D<sup>d</sup> antigen by C1300 cells. As shown in figure 4.25a and 4.26a, a small proportion of untreated C1300 cells expressed low levels of H-2D<sup>d</sup> antigen since the fluorescence of anti-H-2D<sup>d</sup> stained cells was slightly higher than for the same cells stained with FITC conjugated antibody alone (background fluorescence). Statistical analysis of the fluorescence data showed that at least 5% of untreated C1300 cells displayed H-2D<sup>d</sup> antigen on the cell surface. IFN- $\alpha\beta$  treatment markedly increased H-2D<sup>d</sup> antigen expression on C1300 cells (fig 4.25a and b). The augmentation of H-2D<sup>d</sup> antigen was first observed at a concentration of 0.1 u/ml IFN- $\alpha\beta$  on at least 5% of C1300 cells, and increased thereafter in a dose-dependent manner (with the exception of 1 u/ml IFN- $\alpha\beta$  which increased H-2D<sup>d</sup> antigen expression to the same level as 0.1 u/ml IFN- $\alpha\beta$ ). Statistical analysis showed that at least 92% of C1300 cells expressed H-2D<sup>d</sup> antigen when treated with 1000 u/ml IFN- $\alpha\beta$ , and that this concentration of IFN- $\alpha\beta$  increased H-2D<sup>d</sup> antigen on at least 88% of cells. IFN- $\gamma$  treatment also markedly increased H-2D<sup>d</sup> antigen expression on C1300 cells (fig 4.26a and b). The augmentation of H-2D<sup>d</sup> antigen by IFN- $\gamma$  was first observed at a concentration of 0.01 u/ml on at least 12% of C1300 cells, and increased thereafter in a dose-dependent manner upto 100 u/ml (with the exception of 0.1 u/ml IFN- $\gamma$  which increased H-2D<sup>d</sup> antigen expression to the same level as 0.01 u/ml IFN- $\gamma$ ). The fluorescence of C1300 cells treated with 1000 u/ml IFN- $\gamma$  was identical to that for C1300 cells treated with 100 u/ml IFN- $\gamma$ , suggesting that the cells were expressing maximum levels of H-2D<sup>d</sup> antigen (data not shown). Statistical analysis showed that at least 84% of C1300 cells expressed H-2D<sup>d</sup> antigen when treated with 100 u/ml IFN- $\gamma$ , and that this concentration of IFN- $\gamma$  increased H-2D<sup>d</sup> antigen on at least 83% of the cells. Figures 4.27 and 4.28 show the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of class I MHC H-2K<sup>k</sup> antigen by C1300 cells. As shown

Figure 4.25

Effect of IFN- $\alpha$  on expression of class I H-2D<sup>d</sup> antigen by C1300 cells



**Key**

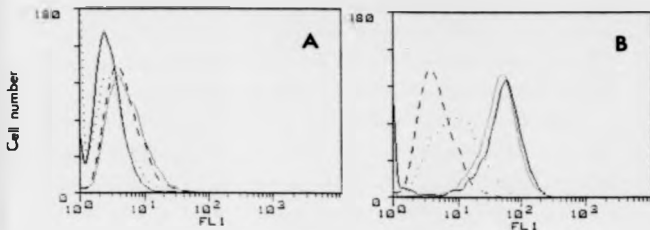
- (A) spaced dots : background fluorescence  
 solid line : untreated  
 close dots : 0.1 u/ml IFN- $\alpha$   
 dashed line : 1 u/ml IFN- $\alpha$   
 (B) dashed line : 1 u/ml IFN- $\alpha$   
 spaced dots : 10 u/ml IFN- $\alpha$   
 close dots : 100 u/ml IFN- $\alpha$   
 solid line : 1000 u/ml IFN- $\alpha$

FL1; Fluorescence intensity channel

- (i) C1300 cells were treated for 48 hours with the appropriate concentration of IFN- $\alpha$  prior to staining H-2D<sup>d</sup> antigen by indirect immunofluorescence as described in methods section H.  
 (ii) 5000 cells analysed in each case.

Figure 4.26

Effect of IFN- $\gamma$  on expression of class I H-2D<sup>d</sup> antigen by C1300 cells



**Key**

- (A) solid line : background fluorescence  
spaced dots : untreated  
close dots : 0.01 u/ml IFN- $\gamma$   
dashed line : 0.1 u/ml IFN- $\gamma$   
(B) dashed line : 0.1 u/ml IFN- $\gamma$   
spaced dots : 1 u/ml IFN- $\gamma$   
close dots : 10 u/ml IFN- $\gamma$   
solid line : 100 u/ml IFN- $\gamma$

FLI; Fluorescence intensity channel

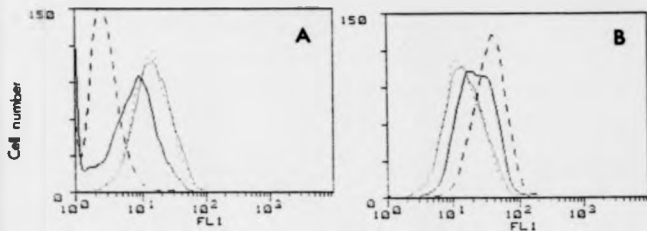
- (i) C1300 cells were treated for 48 hours with the appropriate concentration of IFN- $\gamma$  prior to staining H-2D<sup>d</sup> antigen by indirect immunofluorescence.  
(ii) 5000 cells analysed in each case.

in figure 4.27a and 4.28a, a proportion of untreated C1300 cells expressed moderate levels of H-2K<sup>k</sup> antigen since the fluorescence of anti-H-2K<sup>k</sup> stained cells was clearly higher than for the same cells stained with FITC conjugated antibody alone (background fluorescence). Statistical analysis showed that at least 47% of untreated C1300 cells displayed H-2K<sup>k</sup> antigen on the cell surface. IFN- $\alpha\beta$  treatment markedly increased H-2K<sup>k</sup> antigen expression on C1300 cells (fig 4.27a and b). The augmentation of H-2K<sup>k</sup> antigen expression on IFN- $\alpha\beta$  was first observed at 0.1 u/ml IFN- $\alpha\beta$  on at least 20% of C1300 cells. 1 u/ml and 10 u/ml IFN- $\alpha\beta$  increased H-2K<sup>k</sup> antigen expression to the same level as for 0.1 u/ml IFN- $\alpha\beta$ . Concentrations of IFN- $\alpha\beta$  above 10 u/ml increased H-2K<sup>k</sup> antigen expression on C1300 cells in a dose-dependent manner. Statistical analysis showed that at least 94% of C1300 cells expressed H-2K<sup>k</sup> antigen when treated with IFN- $\alpha\beta$ , and that this concentration of IFN- $\alpha\beta$  increased H-2K<sup>k</sup> antigen on at least 66% of cells. IFN- $\gamma$  also markedly increased H-2K<sup>k</sup> antigen expression on C1300 cells (fig 4.28a and b). The augmentation of H-2K<sup>k</sup> antigen by IFN- $\gamma$  was first observed at a concentration of 0.01 u/ml on at least 38% of C1300 cells, and increased thereafter in a dose-dependent manner up to 10 u/ml IFN- $\gamma$  (with the exception of 0.1 u/ml IFN- $\gamma$  which increased H-2K<sup>k</sup> antigen expression to the same level as 0.01 u/ml IFN- $\gamma$ ). The fluorescence of C1300 cells treated with 1000 u/ml IFN- $\gamma$  was identical to that for C1300 cells treated with 10 u/ml and 100 u/ml IFN- $\gamma$ , suggesting that these cells were expressing maximum levels of H-2K<sup>k</sup> antigen (data not shown). Statistical analysis showed that at least 83% of C1300 cells expressed H-2K<sup>k</sup> antigen when treated with 1000 u/ml IFN- $\gamma$ , and that this concentration of IFN- $\gamma$  increased H-2K<sup>k</sup> antigen on at least 77% of cells.

C1300 cells are derived from the recombinant A/J mouse (H-2<sup>a</sup>) which encodes the class I MHC determinants H-2D<sup>d</sup> and H-2K<sup>k</sup>. By using alloreactive CTL

Figure 4.27

Effect of IFN- $\alpha\beta$  on expression of class I MHC H-2K<sup>b</sup> antigen by C1300 cells



**Key**

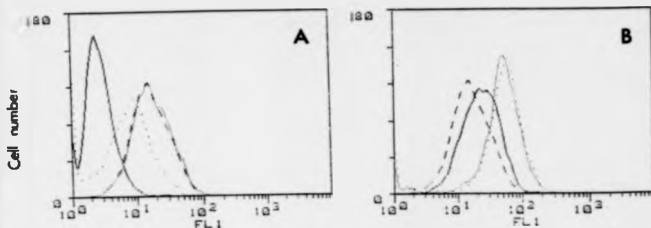
- (A) dashed line : background fluorescence  
 solid line : untreated  
 spaced dots : 0.1 u/ml IFN- $\alpha\beta$   
 close dots : 1 u/ml IFN- $\alpha\beta$
- (B) close dots : 1 u/ml IFN- $\alpha\beta$   
 spaced dots : 10 u/ml IFN- $\alpha\beta$   
 solid line : 100 u/ml IFN- $\alpha\beta$   
 dashed line : 1000 u/ml IFN- $\alpha\beta$

FL1; Fluorescence intensity channel

- (1) C1300 cells were treated for 48 hours with the appropriate concentration of IFN- $\alpha\beta$  prior to staining H-2K<sup>b</sup> antigens by indirect immunofluorescence as described in methods section M.
- (11) 5000 cells analysed in each case.

Figure 4.28

Effect of IFN- $\gamma$  on expression of class I MHC H-2K<sup>k</sup> antigen by C1300 cells



Key

- (A) solid line : background fluorescence  
spaced dots : untreated  
close dots : 0.01 u/ml IFN- $\gamma$   
dashed line : 0.1 u/ml IFN- $\gamma$
- (B) dashed line : 0.1 u/ml IFN- $\gamma$   
solid line : 1 u/ml IFN- $\gamma$   
close dots : 10 u/ml IFN- $\gamma$   
spaced dots : 100 u/ml IFN- $\gamma$

FLI; Fluorescence intensity channel

- (i) C1300 cells were treated for 48 hours with the appropriate concentration of IFN- $\gamma$  prior to staining H-2K<sup>k</sup> antigen by indirect immunofluorescence as described in methods section H.
- (ii) 5000 cells analysed in each case.

specific for H-2<sup>d</sup> and H-2<sup>k</sup> antigens it was possible to study independently the level of functional H-2D<sup>d</sup> and H-2K<sup>k</sup> antigen expressed on C1300 cells, and the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on expression of these antigens. Figure 4.29a shows the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on the susceptibility of C1300 cells to lysis by H-2<sup>d</sup>-anti-H-2<sup>k</sup> alloreactive CTL. It can be seen that untreated C1300 cells were clearly more susceptible to alloreactive CTL lysis than untreated control cells of the wrong haplotype (F815 cells, H-2<sup>d</sup>). Although it is possible that C1300 cells and F815 cells differ in the background level of susceptibility to alloreactive CTL lysis, this observation does at least indicate that some C1300 cells may express a sufficient level of functional class I MHC H-2K<sup>k</sup> antigen for T-cell recognition to occur. IFN- $\alpha\beta$  and IFN- $\gamma$  treatment of C1300 cells increased the susceptibility to alloreactive CTL lysis, indicating that both IFN types increased the ability of C1300 cells to participate in H-2K region associated T-cell mediated immune reactions. This increased ability may be due to an increased level of functional H-2K<sup>k</sup> antigen and/or adhesion molecule display on the cells. The killing by the alloreactive CTL was H-2K<sup>k</sup> specific since L929 cells (H-2<sup>k</sup>) were lysed whereas F815 cells (H-2<sup>d</sup>) were not. Figure 4.29b shows the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on the susceptibility of C1300 cells to lysis by H-2<sup>k</sup>-anti-H-2<sup>d</sup> alloreactive CTL. It can be seen that untreated C1300 cells were clearly more susceptible to alloreactive CTL lysis than untreated control cells of the wrong haplotype (L929 cells, H-2<sup>k</sup>). Again, although the possibility exists that C1300 cells and L929 cells differ in the background level of susceptibility to alloreactive CTL lysis, this observation does at least indicate that some untreated C1300 cells express a sufficient level of functional class I MHC H-2D<sup>d</sup> antigen for T-cell recognition to occur. IFN- $\alpha\beta$  and IFN- $\gamma$  treatment of C1300 cells increased the susceptibility to alloreactive CTL lysis, indicating that both IFN



Figure 4.29

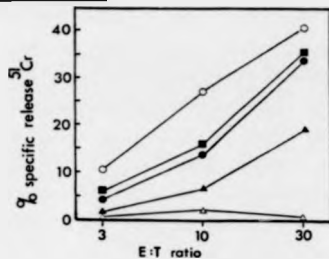
Effect of IFN- $\gamma$  and IFN- $\alpha\beta$  treatment on susceptibility of C1300 cells to lysis by alloreactive CTL

Key

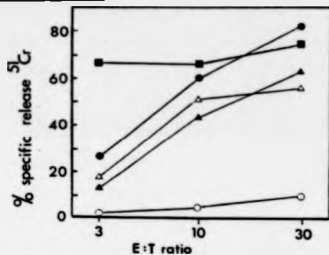
- ( $\blacktriangle$ — $\blacktriangle$ ) C1300 cells (H-2<sup>a</sup>; K<sup>k</sup> D<sup>d</sup>), untreated  
( $\bullet$ — $\bullet$ ) C1300 cells (H-2<sup>a</sup>; K<sup>k</sup> D<sup>d</sup>), treated with 1000 units/ml IFN- $\gamma$   
( $\blacksquare$ — $\blacksquare$ ) C1300 cells (H-2<sup>a</sup>; K<sup>k</sup> D<sup>d</sup>), treated with 1000 units/ml IFN- $\alpha\beta$   
( $\triangle$ — $\triangle$ ) P815 cells (H-2<sup>d</sup>; K<sup>d</sup> D<sup>d</sup>), untreated  
( $\circ$ — $\circ$ ) L929 cells (H-2<sup>k</sup>; K<sup>k</sup> D<sup>k</sup>), untreated  
( $\square$ — $\square$ ) EL4 cells (H-2<sup>b</sup>; K<sup>b</sup> D<sup>b</sup>), untreated

- (i) Cells were treated with IFN- $\alpha\beta$ , IFN- $\gamma$  or medium alone for 48 hours. The cells were then harvested and susceptibility to (a) H2<sup>d</sup>-anti-H2<sup>k</sup>, (b) H2<sup>k</sup>-anti-H2<sup>d</sup> and (c) H2<sup>d</sup>-anti-H2<sup>b</sup> alloreactive CTL lysis measured in a 4.5 hr chromium release assay, as described in methods section M.
- (ii) 'Effector' alloreactive CTL were: Balb/c anti C3H/He (H2<sup>d</sup>-anti-H2<sup>k</sup>)  
C3H/He anti Balb/c (H2<sup>k</sup>-anti-H2<sup>d</sup>)  
Balb/c anti C57 BL/6 (H2<sup>d</sup>-anti-H2<sup>b</sup>)
- (iii) E:T; Effector to Target ratio.

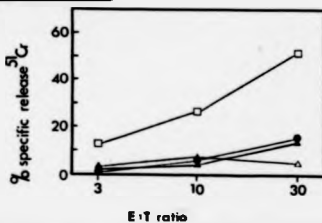
(a)  $H2^d$  anti  $H2^k$



(b)  $H2^k$  anti  $H2^d$



(c)  $H2^d$  anti  $H2^b$

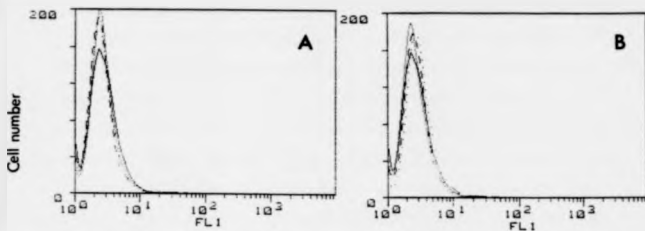


types increased the ability of C1300 cells to participate in H-2D region associated T-cell mediated immune reactions. This increased ability may be due to an increased level of functional H-2D<sup>d</sup> antigen and/or adhesion molecule display on the cells. The killing by the alloreactive CTL was H-2D<sup>d</sup> specific since P815 cells (H-2<sup>d</sup>) were lysed whereas L929 cells (H-2<sup>k</sup>) were not. Figure 4.29c shows the effect of IFN- $\gamma$  on the susceptibility of C1300 cells to lysis by H-2<sup>d</sup>.anti-H-2<sup>b</sup> alloreactive CTL. It can be seen that untreated and IFN- $\gamma$  treated C1300 cells were not susceptible to H-2<sup>d</sup>.anti-H-2<sup>b</sup> alloreactive CTL lysis, since the amounts of chromium released only corresponded to the background lysis observed for untreated control cells of the wrong haplotype (P815 cells, H-2<sup>d</sup>). The susceptibility of IFN- $\alpha\beta$  treated C1300 cells to lysis by H-2<sup>d</sup>.anti-H-2<sup>b</sup> alloreactive CTL was not determined. In contrast to untreated and IFN- $\gamma$  treated C1300 cells, EL4 cells (H-2<sup>b</sup>) were lysed by the alloreactive CTL indicating that the killing was H-2<sup>b</sup> specific.

Figure 4.30 shows the effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on expression of class II MHC H-2A<sup>k</sup> antigen by C1300 cells. As shown in figure 4.30a, untreated C1300 cells displayed no detectable H-2A<sup>k</sup> antigen since the fluorescence of anti-H-2A<sup>k</sup> stained cells was identical to that of the same cells stained with FITC-conjugated antibody alone (background fluorescence). Statistical analysis of the fluorescence data also showed that 0% of untreated C1300 cells displayed H-2A<sup>k</sup> antigen on the cell surface. Neither IFN- $\alpha\beta$  nor IFN- $\gamma$  treatment had any effect upon H-2A<sup>k</sup> antigen expression since the fluorescence of IFN-treated C1300 cells was identical to that for untreated C1300 cells. In a parallel control experiment IFN- $\gamma$  augmented the expression of H-2A<sup>k</sup> antigen on astrocytes (data not shown).

Figure 4.30

Effect of IFN- $\alpha$  and IFN- $\gamma$  on expression of class II MHC H-2A<sup>k</sup> antigen by C1300 cells



Key

- (A) solid line : background fluorescence  
spaced dots : untreated  
close dots : 100 u/ml IFN- $\alpha$   
dashed line : 1000 u/ml IFN- $\alpha$
- (B) solid line : background fluorescence  
spaced dots : untreated  
close dots : 100 u/ml IFN- $\gamma$   
dashed line : 1000 u/ml IFN- $\gamma$

FLI; Fluorescence intensity channel

- (i) C1300 cells were treated for 48 hours with the appropriate concentration of IFN- $\alpha$  or IFN- $\gamma$  prior to staining H-2A<sup>k</sup> antigen by indirect immunofluorescence as described in methods section H.
- (ii) 5000 cells analysed in each case.

Interaction of IFNs  $\alpha$ ,  $\beta$  and  $\gamma$  in the induction of MHC antigen on astrocytes

In the previous section the effects of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  on class I and class II MHC antigen expression on astrocytes were described. In this section the way in which natural IFN- $\alpha\beta$ , recombinant IFN- $\beta$  and recombinant IFN- $\gamma$  interact in the induction of class I (H-2K<sup>b</sup>) and class II (H-2A<sup>k</sup>) MHC antigen on astrocytes is investigated. Again, the expression of MHC antigen on astrocytes was assessed by indirect immunofluorescence staining with quantification by flow cytometry. Table 4.2 shows the way in which IFN- $\alpha\beta$  and IFN- $\gamma$  interact in the induction of class I MHC H-2K<sup>b</sup> antigen on astrocytes. It can be seen that the mean fluorescence for astrocytes treated with concentrations of IFN- $\alpha\beta$  together with IFN- $\gamma$  were greater than for astrocytes treated with the same concentration of either IFN alone, indicating an additive effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on induction of H-2K<sup>b</sup> antigen on astrocytes. The data presented in table 4.2 also clearly show that IFN- $\gamma$  was far more potent (in terms of antiviral activity units) in augmenting H-2K<sup>b</sup> antigen expression on astrocytes than IFN- $\alpha\beta$ , since the mean fluorescence for astrocytes treated with 1 u/ml IFN- $\gamma$  at 462 was clearly higher than for astrocytes treated with 1000 u/ml IFN- $\alpha\beta$  at 326. Similarly, table 4.3 shows the way in which IFN- $\beta$  and IFN- $\gamma$  interact in the induction of class I H-2K<sup>b</sup> antigen on astrocytes. It can be seen that the mean fluorescence for astrocytes treated with concentrations of IFN- $\beta$  together with IFN- $\gamma$  was greater than for astrocytes treated with the same concentration of either IFN alone, indicating an additive effect of IFN- $\beta$  and IFN- $\gamma$  on induction of H-2K<sup>b</sup> antigen on astrocytes. The data presented in table 4.3 also show that IFN- $\gamma$  was more potent (in terms of antiviral activity units) in increasing H-2K<sup>b</sup> antigen expression on astrocytes than IFN- $\beta$ , since the mean

Table 4.2

Effect of IFN- $\alpha\beta$  on the augmentation by IFN- $\gamma$  of class I MHC H-2K<sup>k</sup> antigen on astrocytes

	UNT <sup>a</sup>	10 $\alpha\beta$ <sup>c</sup>	100 $\alpha\beta$	1000 $\alpha\beta$
UNT <sup>b</sup>	11 <sup>d</sup>	30	109	326
1 $\gamma$ <sup>c</sup>	462	541	607	751
10 $\gamma$	862	870	981	1142

Key

- (a) UNT, untreated with IFN- $\alpha\beta$
- (b) UNT, untreated with IFN- $\gamma$
- (c) refer to units/ml IFN- $\alpha\beta$  and IFN- $\gamma$  present
- (d) refer to the mean fluorescence of cells, stained with anti-H-2K<sup>k</sup>

- (i) Astrocytes were treated with either IFN- $\alpha\beta$  alone, IFN- $\gamma$  alone or a combination of IFN- $\alpha\beta$  and IFN- $\gamma$  for 48 hours prior to staining H-2K<sup>k</sup> antigen by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells analysed in each case.
- (iv) All fluorescence histograms obtained followed a normal distribution.

Table 4.3

Effect of IFN- $\beta$  on the augmentation by IFN- $\gamma$  of class I HMG M-2<sup>k</sup> antigen on astrocytes

	UNIT <sup>m</sup>	10 $\beta$ <sup>c</sup>	100 $\beta$	1000 $\beta$
UNT <sup>b</sup>	14 <sup>d</sup>	74	271	629
1 $\gamma$ <sup>c</sup>	398	429	563	812
10 $\gamma$	681	688	896	1041

Key

- (a) UNT, untreated with IFN- $\beta$
  - (b) UNT, untreated with IFN- $\gamma$
  - (c) refer to units/ml IFN- $\beta$  and IFN- $\gamma$  present
  - (d) refer to the mean fluorescence of the cells, stained with anti-M-2K<sup>k</sup>
- (i) Astrocytes were treated with either IFN- $\beta$  alone, IFN- $\gamma$  alone or a combination of IFN- $\beta$  and IFN- $\gamma$  for 48 hours prior to staining M-2K<sup>k</sup> antigen by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells were analysed in each case.
- (iii) All fluorescence histograms obtained followed a normal distribution.

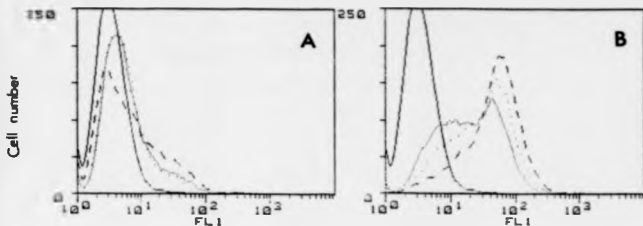
fluorescence for astrocytes treated with 10 u/ml IFN- $\gamma$  at 681 was higher than for astrocytes treated with 1000 u/ml IFN- $\beta$  at 629. The histograms which generated the mean fluorescence values shown in tables 4.2 and 4.3 all followed a normal distribution.

Figure 4.31 shows the way in which IFN- $\alpha\beta$  and IFN- $\gamma$  interact in the induction of class II MHC H-2A<sup>k</sup> antigen on astrocytes. In contrast to induction of class I MHC H-2K<sup>k</sup> antigen in which IFN- $\alpha\beta$  and IFN- $\gamma$  were found to have an additive effect, IFN- $\alpha\beta$  was found to slightly inhibit the ability of IFN- $\gamma$  to augment class II H-2A<sup>k</sup> antigen expression on at least some astrocytes, since the fluorescence of astrocytes treated with both IFN- $\alpha\beta$  and IFN- $\gamma$  was slightly lower than for astrocytes treated with the same concentration of IFN- $\gamma$  alone (fig 4.31a and b). Statistical analysis of the fluorescence data showed that 1 u/ml IFN- $\gamma$  increased H-2A<sup>k</sup> antigen expression on at least 27% of astrocytes, and that this concentration of IFN- $\gamma$  only increased H-2A<sup>k</sup> antigen expression on 19% of astrocytes in the presence of 1000 u/ml IFN- $\alpha\beta$  and on 17% of astrocytes in the presence of 10 000 u/ml IFN- $\alpha\beta$ . Similarly, statistical analysis showed that 10 u/ml IFN- $\gamma$  increased H-2A<sup>k</sup> antigen expression on at least 85% of astrocytes, and that this concentration of IFN- $\gamma$  only increased H-2A<sup>k</sup> antigen expression on 77% of astrocytes in the presence of 1000 u/ml IFN- $\alpha\beta$  and on 67% of astrocytes in the presence of 10 000 u/ml IFN- $\alpha\beta$ . Thus, 1000 u/ml IFN- $\alpha\beta$  and 10 000 u/ml IFN- $\alpha\beta$  inhibited the ability of 10 u/ml IFN- $\gamma$  to augment H-2A<sup>k</sup> antigen on at least 8% and 18% of astrocytes, respectively. To determine whether a longer treatment with IFN- $\alpha\beta$  would have a greater effect on the augmentation by IFN- $\gamma$  of class II H-2A<sup>k</sup> antigen on astrocytes, the cells were pretreated with IFN- $\alpha\beta$  for 2 days prior to the addition of IFN- $\gamma$  and incubation for a further 2 days. As shown in figure 4.32, the effect of IFN- $\alpha\beta$  was essentially no greater. Statistical



Figure 4.11

Effect of IFN- $\alpha$  on the augmentation by IFN- $\gamma$  of class II MHC H-2A<sup>k</sup> antigen on astrocytes



Key

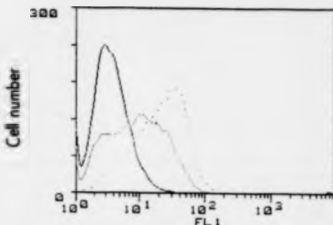
- (A) solid line : untreated  
dashed line : 1 u/ml IFN- $\gamma$  alone  
close dots : 1 u/ml IFN- $\gamma$  + 1000 u/ml IFN- $\alpha$   
spaced dots : 1 u/ml IFN- $\gamma$  + 10000 u/ml IFN- $\alpha$
- (B) solid line : untreated  
dashed line : 10 u/ml IFN- $\gamma$  alone  
spaced dots : 10 u/ml IFN- $\gamma$  + 1000 u/ml IFN- $\alpha$   
close dots : 10 u/ml IFN- $\gamma$  + 10000 u/ml IFN- $\alpha$

FL1; Fluorescence intensity channel

- (i) Astrocytes were treated with IFN- $\gamma$  in the presence or absence of IFN- $\alpha$  for 48 hours prior to staining H-2A<sup>k</sup> antigen by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells analysed in each case.

Figure 4.12

Effect of 2 day pretreatment with IFN- $\alpha\beta$  on the augmentation by IFN- $\gamma$  of class II MHC H-2A<sup>k</sup> antigen on astrocytes



**Key**

solid line : untreated

spaced dots : 10 u/ml IFN- $\gamma$  alone

close dots : 1000 u/ml IFN- $\alpha\beta$  + 10 u/ml IFN- $\gamma$

FLI; Fluorescence intensity channel

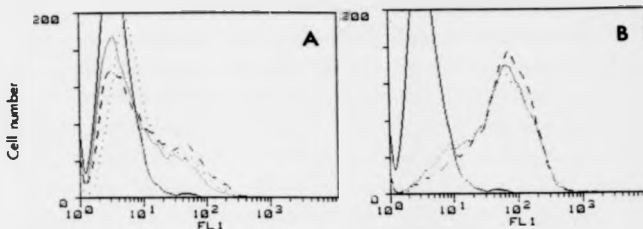
- (i) Astrocytes were treated with 1000 u/ml IFN- $\alpha\beta$  or medium alone for 48 hours prior to the addition of 10 u/ml IFN- $\gamma$  and incubation for a further 48 hours. H-2A<sup>k</sup> antigens were then stained by indirect immunofluorescence as described in methods section H.
- (ii) 10 000 cells analysed in each case.

analysis of the fluorescence data showed that 10 u/ml IFN- $\gamma$  increased H-2A<sup>k</sup> antigen expression on at least 80% of astrocytes, and that this concentration of IFN- $\gamma$  only increased H-2A<sup>k</sup> antigen expression on 69% of astrocytes when pretreated with 1000 u/ml IFN- $\alpha\beta$ . Thus, pretreatment of astrocytes with 1000 u/ml IFN- $\alpha\beta$  inhibited the ability of 10 u/ml IFN- $\gamma$  to augment H-2A<sup>k</sup> antigen expression on 11% of astrocytes, a figure directly comparable to that stated above for 1000 u/ml IFN- $\alpha\beta$  excluding the 48 hour pretreatment prior to IFN- $\gamma$  treatment.

Figure 4.33 shows the way in which IFN- $\beta$  and IFN- $\gamma$  interact in the induction of class II MHC H-2A<sup>k</sup> antigen on astrocytes. Again, in contrast to the induction of class I MHC H-2K<sup>k</sup> antigen in which IFN- $\beta$  and IFN- $\gamma$  were found to have an additive effect, IFN- $\beta$  was found slightly to inhibit the ability of IFN- $\gamma$  to augment class II MHC H-2A<sup>k</sup> antigen on at least some astrocytes, since the fluorescence of astrocytes treated with both IFN- $\beta$  and IFN- $\gamma$  was slightly lower than for astrocytes treated with the same concentration of IFN- $\gamma$  alone (fig 4.33a and b). Statistical analysis showed that 1 u/ml IFN- $\gamma$  increased H-2A<sup>k</sup> antigen expression on at least 34% of astrocytes, and that this concentration of IFN- $\gamma$  only increased H-2A<sup>k</sup> antigen expression on 24% of astrocytes in the presence of 100 u/ml IFN- $\beta$  and on 29% of astrocytes in the presence of 1000 u/ml IFN- $\beta$ . Similarly, statistical analysis showed that 10 u/ml IFN- $\gamma$  increased H-2A<sup>k</sup> antigen expression on at least 83% of astrocytes, and that this concentration of IFN- $\gamma$  only increased H-2A<sup>k</sup> antigen expression on 79% of astrocytes in the presence of 100 u/ml IFN- $\beta$  and on 78% of astrocytes in the presence of 1000 u/ml IFN- $\beta$ . Thus, 100 u/ml IFN- $\beta$  and 1000 units/ml IFN- $\beta$  inhibited the ability of 10 u/ml IFN- $\gamma$  to augment H-2A<sup>k</sup> antigen expression on at least 4% and 5% of astrocytes, respectively. IFN- $\beta$  treatment alone had no effect upon H-2A<sup>k</sup> antigen expression on astrocytes (data not shown).

Figure 4.13

Effect of IFN- $\beta$  on the augmentation by IFN- $\gamma$  of class II MHC H-2A<sup>k</sup> antigen on astrocytes



Key

- (A) solid line : untreated  
dashed line : 1 u/ml IFN- $\gamma$  alone  
close dots : 1 u/ml IFN- $\gamma$  + 100 u/ml IFN- $\beta$   
spaced dots : 1 u/ml IFN- $\gamma$  + 1000 u/ml IFN- $\beta$
- (B) solid line : untreated  
dashed line : 10 u/ml IFN- $\gamma$  alone  
close dots : 10 u/ml IFN- $\gamma$  + 100 u/ml IFN- $\beta$   
spaced dots : 10 u/ml IFN- $\gamma$  + 1000 u/ml IFN- $\beta$

FL1: Fluorescence intensity channel

- (1) Astrocytes were treated with IFN- $\gamma$  in the presence or absence of IFN- $\beta$  for 48 hours prior to staining H-2A<sup>k</sup> antigen by direct immunofluorescence as described in methods section H.
- (11) 10 000 cells analysed in each case.

### Discussion

In this chapter the relative sensitivities of astrocytes, G26-24 oligodendroglioma cells and C1300 neuroblastoma cells to the antiviral effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  are assessed, via the incorporation of [ $^3$ H]uridine into SFV-RNA in IFN-treated SFV-infected cells. It was found that astrocytes and G26-24 cells were highly sensitive to both IFN- $\alpha\beta$  and IFN- $\gamma$ , with total inhibition of SFV-RNA synthesis at 1000 u/ml IFN- $\alpha\beta$  and 100 u/ml IFN- $\gamma$  for astrocytes, and at 300 u/ml IFN- $\alpha\beta$  and 500 u/ml IFN- $\gamma$  for G26-24 cells. Pretreatment of astrocytes with 1000 u/ml IFN- $\alpha\beta$  or 100 u/ml IFN- $\gamma$ , and of G26-24 cells with 500 u/ml IFN- $\gamma$ , prior to infection with SFV, also markedly reduced the amount of SFV antigen displayed on the surface of infected cells. C1300 cells were also found to be highly sensitive to IFN- $\alpha\beta$ , with total inhibition of SFV-RNA synthesis at 1000 u/ml IFN- $\alpha\beta$ . Pretreatment of C1300 cells with 1000 u/ml IFN- $\alpha\beta$ , prior to infection with SFV, markedly reduced the amount of SFV-antigen displayed on the surface of infected cells. In contrast, C1300 cells were found to be relatively insensitive to the antiviral effect of IFN- $\gamma$ , with 2000 u/ml IFN- $\gamma$  inhibiting SFV-RNA synthesis in C1300 cells by only 65%. Similarly, treatment of C1300 cells with 2000 u/ml IFN- $\gamma$ , prior to infection with SFV, reduced SFV antigen display on only a proportion of the cells, with 29% of IFN- $\gamma$  treated SFV-infected C1300 cells still displaying levels of SFV antigen comparable to non-IFN treated SFV-infected cells.

The effects of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  on class I and class II MHC antigen expression by astrocytes, G26-24 cells and C1300 cells were also determined. The data show that 26-66% (range in separate experiments) of untreated astrocytes displayed moderate levels of H-2K<sup>k</sup> antigen, but in

contrast displayed no detectable H-2D<sup>k</sup> antigen. Experiments in which untreated astrocytes were found to be susceptible to alloreactive CTL lysis also suggest that at least some astrocytes display sufficient levels of functional class I MHC antigen for T-cell recognition to occur. The observation that at least some cultured astrocytes express H-2K<sup>k</sup> antigen is consistent with the results of Fontana and coworkers who demonstrated that cultured astrocytes derived from C57 BL/6 mice display H-2K<sup>b</sup> antigen on the cell surface (Fontana *et al.*, 1986). These results do not however reflect the situation *in vivo*, since less than 1% of astrocytes isolated from the brains of newborn CBA mice were found to display H-2K<sup>k</sup> antigen (Wong *et al.*, 1984). The expression of H-2K<sup>k</sup> antigen on cultured astrocytes may be due to the presence of stimulatory components in the culture medium (e.g. from foetal calf serum). That cultured astrocytes do not express detectable levels of H-2D<sup>k</sup> antigen may reflect the fact that the expression of H-2D and H-2K antigens is independently regulated, as has been demonstrated for mouse embryo fibroblasts (King *et al.*, 1985) or may be due to the inability of the anti-H-2D<sup>k</sup> antibody to stain low levels of H-2D<sup>k</sup> antigen to a sufficient level for detection by flow cytometry. Very recently Skias and coworkers also demonstrated that untreated cultured astrocytes derived from DBA/2 mice were susceptible to alloreactive CTL lysis (Skias *et al.*, 1987). The data presented in this chapter also show that IFN- $\alpha$  and IFN- $\gamma$  treatment markedly increased H-2D<sup>k</sup> and H-2K<sup>k</sup> antigen expression on astrocytes, in a dose-dependant manner. Observation of the data obtained also revealed that IFN- $\gamma$  was more potent in inducing class I MHC class I MHC antigen expression than IFN- $\alpha$ , on the basis of antiviral activity units. Thus, it was found that 10 u/ml IFN- $\alpha$  increased H-2D<sup>k</sup> and H-2K<sup>k</sup> antigen expression on 19% and 7% of astrocytes respectively, whereas 0.1 unit/ml IFN- $\gamma$  increased H-2D<sup>k</sup> and H-2K<sup>k</sup> antigen expression on 57% and 62% of astrocytes, respectively. Since the astrocyte cultures are at least

90% GFAP<sup>+</sup> astrocyte in composition (see chapter 3) and greater than 90% of cells displayed H-2D<sup>k</sup> and H-2K<sup>k</sup> antigen when treated with 1000 u/ml IFN- $\alpha\beta$  or 100 u/ml IFN- $\gamma$ , it follows that at least the majority of IFN- $\alpha\beta$  and IFN- $\gamma$  treated astrocytes must have displayed H-2D<sup>k</sup> and H-2K<sup>k</sup> antigen on the cell surface. It is not possible from the data obtained to determine whether IFN- $\alpha\beta$  and IFN- $\gamma$  treatment also increased H-2D<sup>k</sup> and H-2K<sup>k</sup> antigen expression on the (at most) 10% uncharacterised cells present in these cultures. It does however seem to be a strong possibility, since IFN- $\alpha\beta$  has been shown to increase class I MHC antigen expression on fibroblasts (Dr A Morris, University of Warwick, unpublished observations) and IFN- $\gamma$  has been shown to increase class I MHC antigen expression on fibroblasts, neurons, microglial cells and oligodendrocytes (Wong *et al.*, 1984; Suzumura *et al.*, 1987; Maudslay and Morris, 1988). IFN- $\alpha\beta$  and IFN- $\gamma$  treatment also increased the susceptibility of astrocytes to alloreactive CTL lysis, indicating that both IFNs increased the ability of astrocytes to participate in class I MHC restricted T-cell mediated immune reactions. The ability of IFN- $\alpha\beta$  and IFN- $\gamma$  to increase class I MHC H-2K<sup>k</sup> antigen expression on cultured astrocytes, derived from CBA mice, has been reported previously by Wong and coworkers, who also extended their studies *in vivo* and found that intracerebral injection of IFN- $\gamma$  increased H-2K<sup>k</sup> antigen expression on astrocytes within the brain (Wong *et al.*, 1984). In contrast to the results presented in this chapter, Skias and coworkers recently reported that IFN- $\gamma$  treatment did not increase the susceptibility of DBA/2 astrocytes to lysis by an alloreactive anti-H-2L<sup>d</sup> CTL clone (Skias *et al.*, 1987). The reason for this discrepancy in results is not clear since IFN- $\gamma$  treatment increased H-2L<sup>d</sup> antigen expression on DBA/2 astrocytes, as assessed by indirect immunofluorescence staining with analysis by UV microscopy. However, Skias and coworkers also reported that the increase in H-2L<sup>d</sup> antigen expression could not be detected by flow cytometry. This

suggests that treatment with IFN- $\gamma$  may have only very slightly increased H-2L<sup>d</sup> antigen expression on the DBA/2 astrocytes, and perhaps by a level which could not be detected in the alloreactive CTL assay. Alternatively, IFN- $\gamma$  treatment may not increase the level of functional H-2L<sup>d</sup> antigen expression on DBA/2 astrocytes.

The data presented in this chapter also show that untreated astrocytes displayed undetectable levels of class II MHC H-2A<sup>b</sup> antigen on the cell surface. IFN- $\gamma$  treatment induced H-2A<sup>b</sup> antigen expression on astrocytes, but in contrast IFN- $\alpha\beta$  treatment did not. Similarly, IFN- $\gamma$  treatment increased the ability of astrocytes to stimulate an allogeneic mixed lymphocyte reaction (MLR) indicating an increased level of functional class II MHC antigen on the cells. IFN- $\alpha\beta$  treatment had no effect on the ability of astrocytes to stimulate an allogeneic MLR. These observations are consistent with previous reports by Hirsch *et al.* (1983) and Wong *et al.* (1984) which demonstrated that untreated cultured astrocytes (derived from outbred Swiss mice and CBA mice, respectively) displayed undetectable amounts of class II MHC antigen, as assessed by indirect immunofluorescence staining. Furthermore, these groups also reported that IFN- $\gamma$  treatment induced class II MHC antigen on cultured astrocytes, whereas IFN- $\alpha\beta$  treatment did not. That IFN- $\gamma$  has the potential to induce class II MHC antigen display on astrocytes *in vivo* has been shown by Wong and coworkers, who directly injected IFN- $\gamma$  into the brains of CBA mice (Wong *et al.*, 1984). Since the present studies were undertaken, Fontana and coworkers have also demonstrated that IFN- $\gamma$  treatment increases the ability of astrocytes (derived from C57 BL/6 mice) to stimulate an allogeneic MLR (Fontana *et al.* 1986).

In the present study IFN- $\gamma$  treatment was found to induce H-2A<sup>b</sup> antigen



expression on at least 86% of the cells present in the astrocyte cultures. Since the cultures are at least 90% GFAP<sup>+</sup> astrocytes in composition it follows that at least the majority of IFN- $\gamma$  treated astrocytes must have displayed H-2A<sup>k</sup> antigen on the cell surface. It cannot be deduced from the data obtained whether IFN- $\gamma$  treatment also induced H-2A<sup>k</sup> antigen expression on the (at most) 10% uncharacterised cells present in these cultures. However, it does at least seem possible since IFN- $\gamma$  treatment has been shown to augment class II MHC antigen expression on fibroblasts and microglial cells (Maudsley and Morris, 1988; Suzumura *et al.*, 1987). A possible experimental approach to determine the identity of all cells which express (and do not express) H-2A<sup>k</sup> antigen (as well as H-2D<sup>k</sup> and H-2K<sup>k</sup> antigen) could be to use a fluorescence activated cell sorter to sort H-2A<sup>k</sup> expressing cells (and non-H-2A<sup>k</sup> expressing cells) and then use a range of antibodies to stain markers specific for the possible cell types present. For example, antiserum to neurofilaments and the monoclonal antibody A2B5 may be used to characterise neurons, antiserum to galactocerebroside to characterise oligodendrocytes and antiserum to fibronectin to characterise fibroblasts (Mirsky, 1982). Ependymal cells can be recognised by their beating cilia, and microglial cells by non-specific esterase activity, Fc receptor expression and the ability to endocytose latex beads (Mirsky, 1982; Suzumura *et al.*, 1987).

The effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on class I and class II MHC antigen expression by G26-24 oligodendroglioma cells were also determined. The data show that 12-84% and 15-42% (range in separate experiments) of untreated G26-24 cells displayed moderate levels of H-2D<sup>b</sup> and H-2K<sup>b</sup> antigen, respectively. Experiments in which untreated G26-24 cells were found to be susceptible to alloreactive CTL lysis also suggest that at least some G26-24 cells display sufficient levels of functional class I MHC

antigen for T-cell recognition to occur. These observations do not however reflect the situation with normal oligodendrocytes *in vivo*, since less than 1% of oligodendrocytes isolated from the brains of newborn CBA mice were found to display class I MHC antigen (Wong *et al.*, 1984). The expression of H-2D<sup>b</sup> and H-2K<sup>b</sup> antigen on at least some G26-24 cells may be due to the presence of stimulatory components in the culture medium (as was proposed for cultured astrocytes) or may be a feature of the transformed cells. IFN- $\alpha$  and IFN- $\gamma$  treatment markedly increased H-2D<sup>b</sup> and H-2K<sup>b</sup> antigen expression on G26-24 cells, in a dose-dependent manner. Observation of the data obtained also revealed that IFN- $\gamma$  was more potent in inducing class I MHC antigen expression than IFN- $\alpha$ , on the basis of antiviral activity units. Thus, it was found that 1 u/ml IFN- $\alpha$  increased H-2D<sup>b</sup> and H-2K<sup>b</sup> antigen expression on 10% and 3% of G26-24 cells respectively, whereas 0.05 u/ml IFN- $\gamma$  increased H-2D<sup>b</sup> and H-2K<sup>b</sup> antigen expression on 28% and 15% of G26-24 cells, respectively. Greater than 65% of G26-24 cells expressed H-2D<sup>b</sup> and H-2K<sup>b</sup> antigen when treated with 1000 u/ml IFN- $\alpha$ , and greater than 87% when treated with 500 u/ml IFN- $\gamma$ . IFN- $\alpha$  and IFN- $\gamma$  treatment was also found to increase the susceptibility of G26-24 cells to alloreactive CTL lysis, indicating that both IFN types increased the ability of G26-24 cells to participate in class I MHC restricted T-cell mediated immune reactions. The ability of IFN- $\gamma$  to increase class I MHC H-2K<sup>k</sup> antigen expression on normal oligodendrocytes present in 2 day-old mixed brain cell cultures (prepared from CBA mice) has been reported previously by Wong *et al.* (1984). Wong and co-workers also extended their studies *in vivo* and demonstrated that intracerebral administration of IFN- $\gamma$  increased H-2K<sup>k</sup> antigen expression on oligodendrocytes within the brains of CBA mice (Wong *et al.*, 1984).

The data presented in this chapter also show that untreated G26-24 cells

displayed no detectable class II H-2A<sup>b</sup> antigen on the cell surface, as has been shown for normal untreated rat and mouse oligodendrocytes in culture (Lisak *et al.*, 1983; Wong *et al.*, 1984). Whether untreated cultured normal human oligodendrocytes display detectable amounts of class II MHC antigen is still controversial. Thus, Lisak and coworkers (Lisak *et al.*, 1983) reported that untreated cultured human oligodendrocytes (obtained at autopsy from two subjects with no known neurological disease) displayed no detectable class II MHC antigen, whereas Kim and coworkers (Kim *et al.*, 1985) detected class II MHC antigen on the surface of some oligodendrocytes within cultures prepared from six subjects out of a total of twelve studied (all twelve subjects had no known neurological disease at autopsy). That Lisak and coworkers were unable to detect class II MHC antigen on cultured human oligodendrocytes may have been related to the limited number of samples obtained, in that cultures were prepared from only two subjects. In the present study, IFN- $\gamma$  treatment induced H-2A<sup>b</sup> antigen expression on G26-24 cells, but in contrast IFN- $\alpha\beta$  treatment did not. The ability of IFN- $\gamma$  to induce class II MHC antigen expression on G26-24 oligodendroglioma cells does not reflect the situation with normal oligodendrocytes, at least in the murine system. Thus, it has been shown that IFN- $\gamma$  treatment does not increase class II MHC antigen expression on mouse oligodendrocytes in culture (Wong *et al.*, 1984; Suzumura *et al.*, 1986). Similarly, Wong and coworkers also reported that intracerebral administration of IFN- $\gamma$  did not increase class II MHC antigen expression on oligodendrocytes within the brains of CBA mice (Wong *et al.*, 1984). Whether IFN- $\gamma$  augments class II MHC antigen expression on normal human oligodendrocytes remains to be determined.

The effects of IFN- $\alpha\beta$  and IFN- $\gamma$  on class I and class II MHC antigen expression by C1300 neuroblastoma cells were also determined. The data

show that 5% and 47% of untreated C1300 cells displayed moderate levels of H-2D<sup>d</sup> and H-2K<sup>k</sup> antigen, respectively. Experiments in which untreated C1300 cells were found to be susceptible to both H-2<sup>d</sup> and H-2<sup>k</sup> specific alloreactive CTL lysis also suggest that at least some C1300 cells display sufficient levels of functional class I MHC H-2D<sup>d</sup> and H-2K<sup>k</sup> antigen for T-cell recognition to occur. These observations do not reflect the situation with human neuroblastoma cells which have been shown to express exceptionally low levels of class I MHC antigen (Lampson *et al.*, 1983; Lampson and Fisher, 1984) and are not susceptible to alloreactive CTL lysis (Main *et al.*, 1985). These observations also do not reflect the situation with normal murine neurons *in vivo*, since less than 1% of neurons isolated from the brains of newborn CBA mice were found to display class I MHC H-2K<sup>k</sup> antigen (Wong *et al.*, 1984). The expression of H-2D<sup>d</sup> and H-2K<sup>k</sup> antigen on at least some untreated C1300 cells may thereby be a feature unique to the murine C1300 neuroblastoma cell line. IFN- $\alpha$  and IFN- $\gamma$  treatment was also found to markedly increase H-2D<sup>d</sup> and H-2K<sup>k</sup> antigen expression on C1300 cells, in an essentially dose-dependant manner. Observation of the data obtained also again revealed that IFN- $\gamma$  was more potent in inducing class I MHC antigen expression than IFN- $\alpha$ , on the basis of antiviral activity units. Thus, it was found that 100 u/ml IFN- $\alpha$  increased H-2D<sup>d</sup> and H-2K<sup>k</sup> antigen expression on 45% and 35% of C1300 cells respectively, whereas 1 u/ml IFN- $\gamma$  increased H-2D<sup>d</sup> and H-2K<sup>k</sup> antigen expression on 42% and 37% of cells, respectively. These observations contrast with studies on the sensitivity of C1300 cells to the antiviral effect of IFN- $\alpha$  and IFN- $\gamma$ , in which C1300 cells were shown to be sensitive to IFN- $\alpha$ , but relatively insensitive to IFN- $\gamma$ . Thus, IFN- $\gamma$  induces class I MHC antigen expression on C1300 cells at concentrations far lower than needed to induce an antiviral state. Similar observations have also been made previously by Wallach and coworkers who demonstrated that IFN- $\gamma$

induced class I MHC antigen expression on human fibroblast and lymphoblastoid cells at concentrations far lower than needed to induce (2'-5') oligo (A) synthetase and the antiviral state (Wallach *et al.*, 1982). In the present study IFN- $\alpha\beta$  and IFN- $\gamma$  treatment was also found slightly to increase the susceptibility of C1300 cells to H-2<sup>d</sup> and H-2<sup>k</sup> specific alloreactive CTL lysis, indicating that both IFN types increased the ability of C1300 cells to participate in H-2D and H-2K region associated T-cell mediated immune reactions. The ability of IFN- $\gamma$  to augment class I MHC antigen expression on human neuroblastoma cells has also been reported previously by Lampson and Fisher (1984). Similarly, IFN- $\gamma$  has been shown to increase class I MHC antigen expression on at least some normal neurons in mixed brain cell cultures, derived from newborn CBA mice, as well as on neurons within the brains of CBA mice (Wong *et al.*, 1984).

The data presented in this chapter also show that untreated C1300 cells displayed no detectable class II MHC H-2A<sup>k</sup> antigen on the cell surface. Neither IFN- $\alpha\beta$  nor IFN- $\gamma$  treatment of C1300 cells was found to induce H-2A<sup>k</sup> antigen expression. Similarly, Lampson and Fisher (1984) reported that both untreated and IFN- $\gamma$  treated human neuroblastoma cells displayed no detectable class II MHC antigen on the cell surface, and Wong and coworkers demonstrated that intracerebral administration of IFN- $\gamma$  did not induce class II MHC antigen expression on normal neurons within the brains of CBA mice (Wong *et al.*, 1984).

In this chapter the way in which natural IFN- $\alpha\beta$ , recombinant IFN- $\beta$  and recombinant IFN- $\gamma$  interact in the induction of class I and class II MHC antigen expression was also investigated. The data obtained clearly show that IFN- $\alpha\beta$  and IFN- $\beta$  were additive with IFN- $\gamma$  for the enhancement of class I MHC H-2K<sup>k</sup> antigen on astrocytes. Similar observations have also been

made by Lapierre and coworkers who demonstrated that recombinant IFN- $\alpha$  and recombinant IFN- $\beta$  were additive with IFN- $\gamma$  for the enhancement of class I MHC HLA-A,B antigen on human endothelial cells (Lapierre *et al.*, 1988) and by Dr A Morris (University of Warwick) who demonstrated that natural IFN- $\alpha\beta$  and recombinant IFN- $\beta$  were additive with IFN- $\gamma$  for the enhancement of class I MHC H-2D<sup>b</sup> antigen on G26-24 cells, and H-2K<sup>k</sup> antigen on C3H10T<sup>1/2</sup> fibroblast cells (unpublished observations). In contrast to the augmentation of class I MHC antigen expression, IFN- $\alpha\beta$  and IFN- $\beta$  were found to inhibit the ability of IFN- $\gamma$  to augment class II MHC H-2A<sup>k</sup> antigen expression on a proportion of cells present in the astrocyte cultures. The ability of IFN- $\alpha\beta$  to inhibit the augmentation of H-2A<sup>k</sup> antigen expression by IFN- $\gamma$  was dose-dependant, although even very high concentrations of IFN- $\alpha\beta$  only inhibited H-2A<sup>k</sup> antigen expression on a small proportion of cells. Thus, it was found that 10 000 u/ml IFN- $\alpha\beta$  inhibited the ability of 10 u/ml IFN- $\gamma$  to augment H-2A<sup>k</sup> antigen expression on at least 18% of the cells present in the astrocyte cultures. Since the cultures were at least 90% GFAP<sup>+</sup> astrocyte in composition, it follows that 10 000 u/ml IFN- $\alpha\beta$  must have inhibited the ability of IFN- $\gamma$  to augment H-2A<sup>k</sup> antigen expression on at least some astrocytes. It cannot be deduced from the data obtained whether IFN- $\alpha\beta$  also inhibited the ability of IFN- $\gamma$  to augment H-2A<sup>k</sup> antigen expression on the (at most) 10% uncharacterised cells present in these cultures. 1000 u/ml IFN- $\beta$  also inhibited the ability of 10 u/ml IFN- $\gamma$  to augment H-2A<sup>k</sup> antigen expression on at least 5% of the cells present in the astrocyte cultures. Since the cultures are (at least) 90% GFAP<sup>+</sup> astrocyte in composition, it is not possible to determine whether IFN- $\beta$  inhibited the ability of IFN- $\gamma$  to augment H-2A<sup>k</sup> antigen expression on astrocytes or on the uncharacterised cells present in the cultures. The ability of IFN- $\alpha\beta$  and  $\beta$  to inhibit the induction by IFN- $\gamma$  of class II MHC antigen expression has been reported previously in other cell systems. Thus, it has been

demonstrated that natural IFN- $\alpha$ , natural IFN- $\beta$  and recombinant IFN- $\beta$  inhibit the ability of recombinant IFN- $\gamma$  to augment class II MHC antigen expression on macrophages (Ling *et al.*, 1985; Inaba *et al.*, 1986; Fertsch *et al.*, 1987; Kitaura *et al.*, 1988) and hence the ability of IFN- $\gamma$  treated macrophages to stimulate an allogeneic mixed lymphocyte reaction (Inaba *et al.*, 1986). Experiments in which monoclonal antibodies to IFN- $\alpha$  and IFN- $\beta$  were found to neutralise the ability of natural IFN- $\alpha$  and IFN- $\beta$  to inhibit the induction by IFN- $\gamma$  of class II MHC antigen expression also demonstrated that the effects observed were not due to contaminants in the natural IFN samples. Similarly, Lapiere and coworkers demonstrated that recombinant IFN- $\alpha$  and recombinant IFN- $\beta$  inhibit the ability of recombinant IFN- $\gamma$  to augment class II MHC HLA-DR antigen expression on human endothelial cells (Lapiere *et al.*, 1988) and Dr A Morris (University of Warwick) demonstrated that natural IFN- $\alpha\beta$  and recombinant IFN- $\beta$  inhibit the ability of IFN- $\gamma$  to augment H-2A<sup>b</sup> and H-2A<sup>k</sup> antigen expression on G26-24 cells and C3H10T $\frac{1}{2}$  fibroblast cells, respectively (unpublished observations). The mechanisms by which IFN- $\alpha$  and IFN- $\beta$  inhibit the ability of IFN- $\gamma$  to augment class II MHC antigen expression on macrophages has been subject to a number of studies. Thus, it has been shown that IFN- $\alpha$  and IFN- $\beta$  down-regulate the level of IFN- $\gamma$  induced, class II MHC antigen mRNA synthesis in macrophages, in a dose-dependant manner (Fertsch *et al.*, 1987; Kitaura *et al.*, 1988). The down-regulation by IFN- $\beta$  of the level of IFN- $\gamma$  induced class II MHC antigen mRNA synthesis was also found to be sensitive to cycloheximide, an inhibitor of protein synthesis. Since the induction by IFN- $\gamma$  of class II mRNA synthesis was insensitive to cycloheximide, these results suggest that the negative effect of IFN- $\beta$  was mediated by some de novo synthesised protein(s) which interfered with class II MHC antigen mRNA induction by IFN- $\gamma$  (Kitaura *et al.*, 1988). Whether natural IFN- $\alpha\beta$  and recombinant IFN- $\beta$  inhibit the ability of recombinant IFN- $\gamma$  to augment class II MHC antigen

expression on the cells present in the astrocyte cultures by a similar mechanism, remains to be determined.

The potential importance of the effect of IFNs on class I and class II MHC antigen expression lies in the fact that T-cells can only recognise viral antigen which is presented in association with antigen of the MHC (Klein *et al.*, 1981). Since brain cells usually express essentially undetectable amounts of MHC antigen, the augmentation by IFN of class I and class II MHC antigen expression may increase the cytotoxic T-cell and helper T-cell recognition of virus infected cells, and hence play a role in the elimination of virus from the brain. This possibility is investigated further in chapters 5 and 6. Since cytotoxic T-cells and helper T-cells release IFN- $\gamma$  on recognition of viral antigen (Morris *et al.*, 1982; Cunningham *et al.*, 1985) and IFN- $\gamma$  induces class II MHC antigen expression, the existence of a possible feedback loop to amplify T-cell responses is apparent. That IFN- $\alpha\beta$  can at least to some extent inhibit the induction by IFN- $\gamma$  of class II MHC antigen expression suggests a mechanism by which the feedback loop may also be controlled. A problem however is that (in terms of antiviral activity units) a large amount of IFN- $\alpha\beta$  over IFN- $\gamma$  is needed in order to have a significant effect, and in many situations this may not be the case. For example, during an avirulent SFV infection of mice IFN- $\alpha\beta$  is produced within the brain relatively early in infection and reaches maximal levels around day 3 post-infection, after which time the level declines (see chapter 7). In contrast, the ability of T-cells to produce IFN- $\gamma$  develops relatively late in infection and reaches maximal levels at day 7 post-infection (Blackman and Morris, 1984). Thus, when T-cell production of IFN- $\gamma$  reaches maximal levels there may only be low levels of IFN- $\alpha\beta$  present, and probably below the concentration required to inhibit the ability of IFN- $\gamma$  to induce class II MHC antigen expression.



## Chapter 5

### Effect of IFN on the susceptibility of brain cells to SFV-specific cytotoxic T-lymphocyte lysis

As discussed in detail in chapter 1, the importance of MHC antigen expression in the immune recognition of target cells by antigen-specific cytotoxic T-lymphocytes is now well recognised. With few exceptions, CTL only recognise foreign antigen on the surface of a target cell when presented in association with class I MHC antigen. Furthermore, the activity of CTL is restricted to those targets that express the same class I MHC antigen gene products as the effector CTL. Since CTL recognise both foreign antigen and class I MHC antigen on the surface of a target cell, it follows that any factor which modulates the expression of either of these antigens may also modulate the susceptibility of the target cell to CTL lysis.

In chapter 4, data were presented which showed that IFN pretreatment of astrocytes, G26-24 oligodendrogloma cells and C1300 neuroblastoma cells, prior to infection with SFV, markedly reduced the expression of SFV antigen on the surface of the infected cells. In contrast, IFN-treatment was found to markedly increase the expression of class I MHC antigen on the surface of the cells. In this chapter the effect of IFN pretreatment of astrocytes, G26-24 cells and C1300 cells, prior to infection with SFV, on the susceptibility to SFV-specific CTL lysis is investigated.

## Results

### Effect of IFN treatment on susceptibility of astrocytes to SFV-specific cytotoxic T-lymphocyte lysis

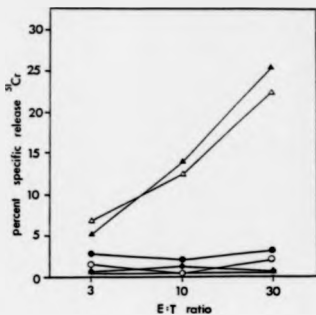
The effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  treatment on the susceptibility of SFV-infected astrocytes to lysis by SFV-specific cytotoxic T-lymphocytes (CTL) and anti-SFV serum in the presence of complement was investigated as described in methods section M. Astrocytes were treated with 1000 u/ml IFN- $\alpha\beta$  or 100 u/ml IFN- $\gamma$  since these concentrations were previously found to strongly augment class I MHC antigen expression, completely inhibit SFV-RNA synthesis and markedly reduce SFV antigen expression on astrocytes (see chapter 4).

Figure 5.1a shows the effect of IFN- $\alpha\beta$  treatment on the susceptibility of SFV-infected astrocytes to SFV-specific CTL lysis. It can be seen that both non-IFN treated SFV-infected astrocytes and IFN- $\alpha\beta$  treated SFV-infected astrocytes were clearly susceptible to lysis by SFV-specific CTL, and that quantitatively similar levels of lysis were observed for each. Uninfected astrocytes (either non-IFN treated or IFN- $\alpha\beta$  treated) and SFV-infected allogeneic EL4 cells (H-2<sup>b</sup>) were not killed, hence the killing by the SFV-specific CTL was both specific and MHC restricted. Yac-1 cells (which are highly susceptible to lysis by natural killer cells: Chervenak and Wolcott, 1988) were not killed, showing that there was no significant natural killer cell activity in the effector population. The susceptibility of the astrocytes to lysis by anti-SFV serum in the presence of complement was also investigated in order roughly to assess the level of SFV antigen displayed on the cells. As shown in figure 5.1b, non-IFN treated SFV-infected astrocytes were highly susceptible to lysis by the

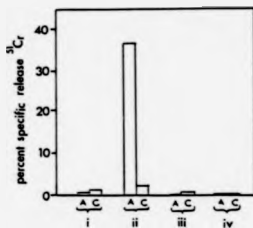
Figure 5.1

Effect of IFN- $\alpha$  on susceptibility of SFV-infected astrocytes to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement







(A) SFV-specific cytotoxic T-lymphocytes



(B) anti-SFV serum in the presence of complement



# Key

- (i), () Astrocytes (H-2<sup>k</sup>) + IFN- $\alpha\beta$  + SFV  
(ii), () Astrocytes (H-2<sup>k</sup>) + SFV  
(iii), () Astrocytes (H-2<sup>k</sup>) + IFN- $\alpha\beta$   
(iv), () Astrocytes (H-2<sup>k</sup>) untreated  
() EL4 cells (H-2<sup>b</sup>) + SFV  
() Yac-1 cells (H-2<sup>a</sup>) untreated

A. lysis by anti-SFV serum in the presence of complement

C. lysis by complement alone

- (i) Cells were treated with 1000 u/ml IFN- $\alpha\beta$  or medium alone for 48 hours prior to infection with SFV. The susceptibility of the cells to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement were then determined, as described in methods section M.
- (ii) SFV-specific cytotoxic T-lymphocytes were derived from C3H/He (H-2<sup>k</sup>) mice as described in methods section J, and were used in a 5 hour chromium release assay.

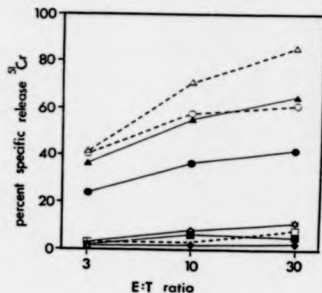
anti-SFV serum in the presence of complement, indicating that the cells were expressing SFV antigen on the cell surface. In contrast, IFN- $\alpha\beta$  treated SFV-infected astrocytes were not susceptible to lysis by the anti-SFV serum in the presence of complement, indicating that IFN- $\alpha\beta$  treatment had markedly reduced SFV antigen expression on the cells (as was shown by indirect immunofluorescence staining, see chapter 4 figure 4.7). Thus, IFN- $\alpha\beta$  treated SFV-infected astrocytes were killed by the SFV-specific CTL to the same level as non-IFN treated SFV-infected astrocytes, despite a marked reduction of SFV antigen expression on the cells. Similar observations were also made in parallel experiments by Mr M J Blackman (currently at the National Institute for Medical Research, London). Mr M J Blackman also demonstrated that SFV infection itself did not increase the non-specific susceptibility of astrocytes to lysis by the SFV-specific CTL, since SFV-infected astrocytes derived from allogeneic DBA/2 mice (H-2<sup>d</sup>) were not killed by the CTL (data not shown).

Figures 5.2a and 5.2b show the effect of IFN- $\gamma$  treatment on the susceptibility of SFV-infected and BPLSFV-treated astrocytes to lysis by SFV-specific CTL and anti-SFV serum in the presence of complement. Again, it can be seen that non-IFN treated SFV-infected astrocytes were highly susceptible to lysis by both SFV-specific CTL and anti-SFV serum in the presence of complement. IFN- $\gamma$  treated SFV-infected astrocytes were found to be markedly more susceptible to SFV-specific CTL lysis than non-IFN treated SFV-infected astrocytes, despite a marked reduction of SFV antigen expression on the cells as assessed by the reduced susceptibility to lysis by anti-SFV serum in the presence of complement (and by indirect immunofluorescence staining, see chapter 4, figure 4.8). Non-IFN treated BPLSFV-treated astrocytes were also clearly susceptible to SFV-specific CTL lysis, although at a lower level than non-IFN treated SFV-infected

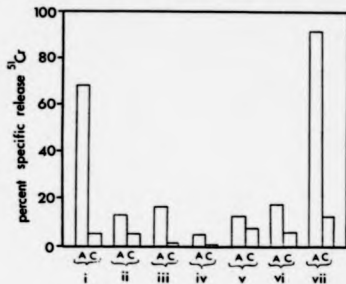
Figure 5.2

Effect of IFN- $\gamma$  on susceptibility of SFV-infected and BPLSFV treated astrocytes to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement

(A) SFV-specific cytotoxic T-lymphocytes



(B) anti-SFV serum in presence of complement



# Key

- (i), (▲—▲) Astrocytes (H-2<sup>k</sup>) + SFV
- (ii), (△-△) Astrocytes (H-2<sup>k</sup>) + IFN-γ + SFV
- (iii), (●—●) Astrocytes (H-2<sup>k</sup>) + BPLSFV
- (iv), (○--○) Astrocytes (H-2<sup>k</sup>) + IFN-γ + BPLSFV
- (v), (■—■) Astrocytes (H-2<sup>k</sup>) untreated
- (vi), (□—□) Astrocytes (H-2<sup>k</sup>) + IFN-γ
- (vii), (◆—◆) ELA cells (H-2<sup>b</sup>) + SFV
- (viii), (⊗—⊗) Yac-1 cells (H-2<sup>d</sup>) untreated

A, lysis by anti-SFV serum in the presence of complement

C, lysis by complement alone

- (i) Cells were treated with 100 u/ml IFN-γ or medium alone for 48 hours prior to infection with SFV or treatment with an equivalent amount of a β-propiolactone inactivated preparation of SFV (BPLSFV). The susceptibility of the cells to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement were then determined, as described in methods section M.
- (ii) SFV-specific cytotoxic T-lymphocytes were derived from C3H/He (H-2<sup>k</sup>) mice as described in methods section J, and were used in a 5 hour chromium release assay.

astrocytes. IFN- $\gamma$  treated BPLSFV-treated astrocytes were markedly more susceptible to SFV-specific CTL lysis than non-IFN treated BPLSFV-treated astrocytes. The susceptibility of non-IFN treated BPLSFV-treated astrocytes and IFN- $\gamma$  treated BPLSFV-treated astrocytes to lysis by anti-SFV serum in the presence of complement corresponded to the background level observed for uninfected astrocytes (either non-IFN treated or IFN- $\gamma$  treated) indicating that the cells were not displaying any detectable SFV antigen on the cell-surface (indirect immunofluorescence staining showed that only 4% of non-IFN treated BPLSFV-treated astrocytes displayed SFV antigen, see chapter 4, figure 4.8). Thus, non-IFN treated BPLSFV-treated astrocytes and IFN- $\gamma$  treated BPLSFV-treated astrocytes were susceptible to SFV-specific CTL lysis despite displaying essentially no detectable SFV antigen on the cell surface. Uninfected astrocytes (either non-IFN treated or IFN- $\gamma$  treated) and SFV-infected allogeneic EL4 cells (H-2<sup>b</sup>) were not killed by the SFV-specific CTL, hence the killing was both specific and MHC-restricted. Similarly, Yac-1 cells were not killed, showing that there was no detectable natural killer cell activity in the effector population.

Effect of SFV-infection and BPLSFV-treatment on class I and class II MHC antigen expression by astrocytes

It is now well established that a number of viruses modulate host cell MHC antigen expression, and by a mechanism which is not mediated via IFN. For example, JHM coronavirus has been shown to induce class II MHC antigen expression, and measles virus has been shown to induce both class I and class II MHC antigen expression, on cultured astrocytes derived from newborn Lewis rats (Massa *et al.*, 1986, 1987a). Virus-modulation of host cell MHC antigen expression has also been shown to influence the ability of the host cell to participate in T-cell mediated immune reactions. Thus, adenovirus-



12 has been shown to abolish class I MHC antigen expression on fibroblasts (Schrier *et al.*, 1983) and reduce the susceptibility of the cells to both alloreactive CTL lysis (Andersson *et al.*, 1987) and virus-(influenza)-specific CTL lysis (Yewdell *et al.*, 1988a). Moloney murine leukemia virus has been shown to increase class I MHC antigen expression on fibroblasts, and increase the susceptibility of the cells to alloreactive CTL lysis (Flyer *et al.*, 1985). It was thereby possible that infection with SFV or treatment with BPLSFV modulated the expression of MHC antigen on astrocytes during the cytotoxicity assays described in the previous section, and hence modulated the susceptibility of the cells to SFV-specific CTL lysis. To investigate this possibility astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) were infected with SFV or treated with BPLSFV, and the effect on class I and class II MHC antigen expression assessed by indirect immunofluorescence staining with quantitation by flow cytometry.

The data in table 5.1 show the effect of SFV-infection and BPLSFV-treatment on class I MHC H-2D<sup>k</sup>K<sup>k</sup> antigen expression by astrocytes (at 10 hours post-treatment). It can be seen that SFV-infection had no effect on H-2D<sup>k</sup>K<sup>k</sup> antigen expression by astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) since the mean fluorescence for SFV-infected cells and uninfected cells were essentially identical. BPLSFV treatment of non-IFN treated astrocytes also had no effect on H-2D<sup>k</sup>K<sup>k</sup> antigen expression. In contrast, BPLSFV-treatment of IFN- $\alpha\beta$  treated and IFN- $\gamma$  treated astrocytes very slightly reduced H-2D<sup>k</sup>K<sup>k</sup> antigen expression, with a decrease in mean fluorescence from 104 for non-BPLSFV treated IFN- $\alpha\beta$  treated astrocytes to 89 for BPLSFV-treated IFN- $\alpha\beta$  treated astrocytes, and from 92 for non-BPLSFV treated IFN- $\gamma$  treated astrocytes to 72 for BPLSFV-treated IFN- $\gamma$  treated astrocytes. The decrease in fluorescence was H-2D<sup>k</sup>K<sup>k</sup> specific since the mean fluorescence of BPLSFV-treated IFN- $\alpha\beta$  treated (or IFN- $\gamma$  treated)

Table 5.1

Effect of BPLSFV treatment and SFV infection on class I MHC H-2D<sup>k</sup> antigen expression by astrocytes

	Uninfected	SFV	BPLSFV
Non-IFN treated	31 <sup>a</sup>	38	33
IFN- $\alpha\beta$	104	102	89
IFN- $\gamma$	92	97	72

(a) refer to the mean fluorescence of the cells, stained with anti-H-2D<sup>k</sup>.

- (i) Astrocytes were treated with either medium alone, 1000 u/ml IFN- $\alpha\beta$  or 100 u/ml IFN- $\gamma$  for 48 hours, prior to infection with SFV or treatment with BPLSFV. At 10 hours post-infection H-2D<sup>k</sup> antigens were stained by indirect immunofluorescence, as described in methods section H.
- (ii) 10,000 cells analysed in each case using the FACS440 flow cytometer at the University of Birmingham, operated by Miss A Milner.

astrocytes stained with an irrelevant antibody (anti-H-2K<sup>d</sup>) was identical to that for non-BPLSFV treated IFN- $\alpha$  treated or IFN- $\gamma$  treated) astrocytes stained with the same antibody (data not shown).

The data in table 5.2 show the effect of SFV infection and BPLSFV treatment on class II MHC H-2A<sup>k</sup> antigen expression by astrocytes. It can be seen that SFV-infection and BPLSFV-treatment had no effect on H-2A<sup>k</sup> antigen expression by non-IFN treated and IFN- $\alpha$  treated astrocytes, since the mean fluorescence of uninfected cells was essentially identical to the mean fluorescence for SFV-infected and BPLSFV-treated cells. SFV-infection and BPLSFV treatment of IFN- $\gamma$  treated astrocytes very slightly reduced H-2A<sup>k</sup> antigen expression, with a decrease in mean fluorescence from 97 for uninfected IFN- $\gamma$  treated astrocytes to 86 for SFV-infected IFN- $\gamma$  treated astrocytes and to 78 for BPLSFV-treated IFN- $\gamma$  treated astrocytes. The decrease in fluorescence was H-2A<sup>k</sup> specific since the mean fluorescence for SFV-infected and BPLSFV-treated IFN- $\gamma$  treated astrocytes stained with an irrelevant antibody (anti-H-2K<sup>d</sup>) were identical to that for uninfected IFN- $\gamma$  treated astrocytes stained with the same antibody (data not shown). Astrocytes were also treated with BPLSFV for a longer period of time in order to determine whether a greater effect on H-2A<sup>k</sup> antigen expression would be observed. The data in figure 5.3 show the effect of 4 day BPLSFV treatment on class II MHC H-2A<sup>k</sup> antigen expression by non-IFN treated astrocytes. It can be seen that 4-day BPLSFV treatment of non-IFN treated astrocytes very slightly increased H-2A<sup>k</sup> antigen expression on astrocytes, since the fluorescence of BPLSFV-treated non-IFN treated astrocytes was slightly higher than for non-BPLSFV treated non-IFN treated astrocytes. However, this increase in fluorescence was not specific for staining H-2A<sup>k</sup> antigen, since the fluorescence of BPLSFV-treated astrocytes stained with an irrelevant antibody (anti-H-2K<sup>d</sup>) was also slightly higher than for non-

Table 5.2

Effect of BPLSFV treatment and SFV infection on class II H-2A<sup>k</sup> antigen expression by astrocytes

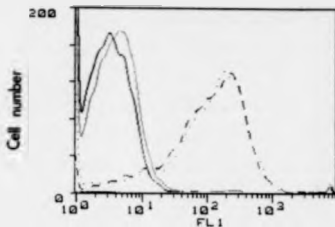
	Uninfected	SFV	BPLSFV
Non-IFN treated	24 <sup>a</sup>	22	21
IFN- $\alpha\beta$	17	18	18
IFN- $\gamma$	97	86	78

(a) refer to the mean fluorescence of the cells, stained with anti-H-2A<sup>k</sup>.

- (i) Astrocytes were treated with either medium alone, 1000 u/ml IFN- $\alpha\beta$  or 100 u/ml IFN- $\gamma$  for 48 hours, prior to infection with SFV or treatment with BPLSFV. At 10 hours post-infection H-2A<sup>k</sup> antigen was stained by indirect immunofluorescence, as described in methods section H.
- (ii) 10 000 cells analysed in each case using the FACS440 flow cytometer at the University of Birmingham, operated by Miss A Milner.

Figure 5.3

Effect of 4-day treatment of astrocytes with BPLSFV on class II MHC H-2A<sup>k</sup> antigen expression



Key

- solid line : Non-IFN treated, non-BPLSFV treated
- spaced dots : IFN- $\gamma$  treated, non-BPLSFV treated
- close dots : Non-IFN treated, BPLSFV treated
- dashed line : IFN- $\gamma$  treated, BPLSFV treated

FL1; Fluorescence intensity channel

- (i) Astrocytes were treated for four days with 5 ml medium either (i) alone, (ii) supplemented with 100 u/ml IFN- $\gamma$ , (iii) supplemented with a 1:5 dilution of BPLSFV (see methods section B), (iv) supplemented with a 1:5 dilution of BPLSFV and 100 u/ml IFN- $\gamma$ .
- (ii) H-2A<sup>k</sup> antigen was stained by indirect immunofluorescence and quantitated by flow cytometry as described in methods section H.
- (iii) 10 000 cells analysed in each case.

BPLSFV treated astrocytes stained with the same antibody (data not shown). Four day BPLSFV-treatment of IFN- $\gamma$  treated astrocytes also had no effect on H-2A<sup>k</sup> antigen expression, since the fluorescence of BPLSFV-treated IFN- $\gamma$  treated astrocytes was identical to that for non-BPLSFV treated IFN- $\gamma$  treated astrocytes.

Effect of IFN treatment on susceptibility of G26-24 cells to SFV-specific cytotoxic T-lymphocyte lysis

The effect of recombinant IFN- $\gamma$  treatment on the susceptibility of SFV-infected G26-24 oligodendroglioma cells to lysis by SFV-specific CTL and anti-SFV serum in the presence of complement were investigated. G26-24 cells were treated with 500 u/ml IFN- $\gamma$  since this concentration was previously found strongly to augment class I MHC antigen expression, completely inhibit SFV-RNA synthesis and markedly reduce SFV antigen expression on G26-24 cells (see chapter 4).

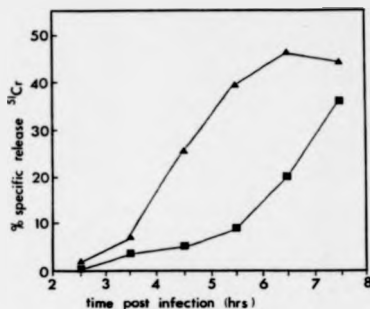
In a preliminary series of experiments (carried out in exactly the same manner as for the astrocyte SFV-specific CTL assays) the ratio of maximum release to spontaneous release of <sup>51</sup>chromium for SFV-infected G26-24 cells was found to be less than 2:1. Ratios this low make calculations of the percent specific release of <sup>51</sup>chromium value unreliable (typically ratios between 5:1 and 10:1 were obtained for data presented in this thesis). The spontaneous release of <sup>51</sup>chromium from SFV-infected G26-24 cells and susceptibility to lysis by anti-SFV serum in the presence of complement was therefore monitored at various times post-infection, in order to determine whether there was a time period during which SFV-infected G26-24 cells display SFV-antigen on the cell surface (hence may be susceptible to SFV-specific CTL lysis) and spontaneously release only low levels of

<sup>51</sup>chromium. This time period could then be used for the co-culture of SFV-infected G26-24 cells with SFV-specific CTL during subsequent cytotoxicity assays and more reliable data would be obtained. As shown in figure 5.4, SFV-infected G26-24 cells became clearly susceptible to lysis by anti-SFV serum in the presence of complement at 44 hours post-infection, and the spontaneous release of <sup>51</sup>chromium did not rise above 20% (which gives a maximum release to spontaneous release value of 3:1) until 64 hours post-infection (it is also interesting to note that the high spontaneous release of <sup>51</sup>Cr from SFV-infected cells also parallels the development of a rapid cytopathic effect, see chapter 3). A further SFV-specific CTL assay with G26-24 cells was thereby carried out and harvested at 64 hours post-infection (astrocyte SFV-specific CTL assays were typically harvested between 9 and 10 hours post-infection).

Figures 5.5a and 5.5b show the effect of IFN- $\gamma$  treatment on the susceptibility of SFV-infected and BPLSFV-treated G26-24 cells to lysis by SFV-specific CTL and anti-SFV serum in the presence of complement. It can be seen that non-IFN treated SFV-infected G26-24 cells were clearly susceptible to lysis by both SFV-specific CTL and anti-SFV serum in the presence of complement. IFN- $\gamma$  treated SFV-infected G26-24 cells were very markedly more susceptible to SFV-specific CTL lysis than non-IFN treated SFV-infected G26-24 cells, despite a marked reduction of SFV antigen expression on the cells as assessed by the reduced susceptibility to lysis by anti-SFV serum in the presence of complement (and by indirect immunofluorescence staining, see chapter 4 figure 4.9). Non-IFN treated BPLSFV-treated G26-24 cells were not specifically susceptible to SFV-specific CTL lysis, since the level of lysis corresponded to that for uninfected G26-24 cells (either non-IFN treated or IFN- $\gamma$  treated). In contrast, IFN- $\gamma$  treated BPLSFV-treated G26-24 cells were very markedly

Figure 5.4

Spontaneous release of  $^{51}\text{Cr}$  from SFV-infected G26-24 cells and lysis by anti-SFV serum in the presence of complement



(▲—▲) Lysis by anti-SFV serum in the presence of complement

(■—■) Spontaneous release of  $^{51}\text{Cr}$

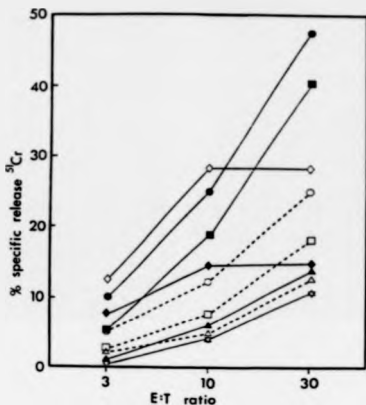
- (1) G26-24 cells were labelled with  $^{51}\text{Cr}$  overnight, infected with SFV, and the susceptibility to lysis by anti-SFV serum in the presence of complement and the spontaneous release of  $^{51}\text{Cr}$  measured at various times post-infection, as described in methods section M.



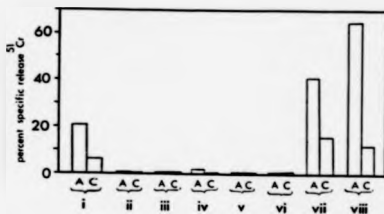
Figure 5.5

Effect of IFN- $\gamma$  on susceptibility of SFV-infected and BLISFV  
created G26-26 cells to lysis by SFV specific cytotoxic  
T-lymphocytes and anti-SFV serum in the presence of complement

(A) SFV-specific cytotoxic T-lymphocytes



(B) anti-SFV serum in presence of complement



# Key

- (i), (○—○) G26-24 cells (H-2<sup>b</sup>) + SFV
- (ii), (●—●) G26-24 cells (H-2<sup>b</sup>) + IFN-γ + SFV
- (iii), (□—□) G26-24 cells (H-2<sup>b</sup>) + BPLSFV
- (iv), (■—■) G26-24 cells (H-2<sup>b</sup>) + IFN-γ + BPLSFV
- (v), (△—△) G26-24 cells (H-2<sup>b</sup>) untreated
- (vi), (▲—▲) G26-24 cells (H-2<sup>b</sup>) + IFN-γ
- (vii), (◇—◇) EL4 cells (H-2<sup>b</sup>) + SFV
- (viii), (◆—◆) RDM4 cells (H-2<sup>k</sup>) + SFV
- (ix), (⊕—⊕) Yac-1 cells (H-2<sup>a</sup>) untreated

A, lysis by anti-SFV serum in the presence of complement

C, lysis by complement alone

- (i) Cells were treated with 500 u/ml IFN-γ or medium alone for 48 hours prior to infection with SFV or treatment with an equivalent amount of a β-propiolactone inactivated preparation of SFV (BPLSFV). The susceptibility of the cells to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement were then determined, as described in methods section H.
- (ii) SFV-specific cytotoxic T-lymphocytes were derived from C57 BL/6 (H-2<sup>b</sup>) mice as described in methods section J, and were used in a 34 hour chromium release assay.

susceptible to SFV-specific CTL lysis, despite displaying no detectable SFV antigen on the cell surface as determined by the non-susceptibility to lysis by anti-SFV serum in the presence of complement (indirect immunofluorescence staining demonstrated that only 3% of IFN- $\gamma$  treated BPLSFV-treated G26-24 cells displayed SFV antigen on the cell surface, see chapter 4 figure 4.9). Uninfected G26-24 cells (either non-IFN treated or IFN- $\gamma$  treated) and SFV-infected allogeneic RDM4 cells (H-2<sup>b</sup>) were found to be slightly susceptible to lysis by SFV-specific CTL. Untreated Yac-1 cells showed a similar level of susceptibility to lysis, suggesting that natural killer cells were also present in the effector population. All killing above this background level of natural killer cell cytotoxicity appears to be both specific and MHC restricted, since SFV-infected EL4 cells (H-2<sup>b</sup>) were killed whereas allogeneic SFV-infected allogeneic RDM4 cells (H-2<sup>b</sup>) and uninfected G26-24 cells (either non-IFN treated or IFN- $\gamma$  treated) were not.

#### Effect of IFN-treatment on susceptibility of C1300 cells to SFV-specific cytotoxic T-lymphocyte lysis

It is now well established that for virus-specific CTL lysis to occur, the virus infected target cell and virus specific CTL must share identical class I MHC H-2D or H-2K region products (the phenomenon of MHC restriction: Zinkernagel and Doherty, 1974, 1979). C1300 neuroblastoma cells were derived from the recombinant A/J mouse (H-2<sup>a</sup>) which encodes the class I MHC region alleles H-2D<sup>d</sup> and H-2K<sup>k</sup> (hereafter referred to as D<sup>d</sup>K<sup>k</sup>, or with the different haplotype designations for different cell-types and strains of mice as appropriate). It was thereby reasoned that by using SFV-specific CTL generated in H-2<sup>d</sup> (D<sup>d</sup>K<sup>d</sup>) and H-2<sup>k</sup> (D<sup>k</sup>K<sup>k</sup>) mice, it should be possible independently to study the SFV-specific CTL responses to H-2D<sup>d</sup>

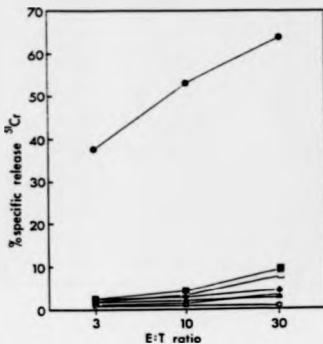
and H-2<sup>k</sup> region products on SFV-infected C1300 cells.

The effect of recombinant IFN- $\gamma$  treatment on the susceptibility of SFV-infected C1300 cells to lysis by SFV-specific CTL (generated in H-2<sup>d</sup> and H-2<sup>k</sup> mice) and anti-SFV serum in the presence of complement were investigated. C1300 cells were treated with 1000 u/ml IFN- $\gamma$  since this concentration was previously found strongly to augment class I MHC antigen expression on C1300 cells, and inhibit both SFV-RNA synthesis and SFV antigen display on at least the majority of SFV-infected C1300 cells (see chapter 4). Figures 5.6a and 5.6b show the effect of IFN- $\gamma$  treatment on the susceptibility of SFV-infected and BPLSFV-treated C1300 cells to lysis by both SFV-specific CTL (derived from C3H/He mice, H-2<sup>k</sup>) and anti-SFV serum in the presence of complement. It can be seen that neither SFV-infected nor BPLSFV-treated C1300 cells (either non-IFN treated or IFN- $\gamma$  treated) were significantly killed by the SFV-specific (H-2<sup>k</sup>) CTL. In contrast, SFV-infected C1300 cells (both non-IFN treated and IFN- $\gamma$  treated) were clearly susceptible to lysis by anti-SFV serum in the presence of complement, indicating that at least some cells were displaying SFV antigen on the cell-surface. Hence, non-IFN treated and IFN- $\gamma$  treated SFV-infected C1300 cells were not susceptible to SFV-specific (H-2<sup>k</sup>) CTL lysis despite displaying SFV antigen (and class I MHC H-2K<sup>b</sup> antigen, see chapter 4 figures 4.28 and 4.29) on the cell surface. SFV-infected L929 cells (H-2<sup>k</sup>) were highly susceptible to SFV-specific (H-2<sup>k</sup>) CTL lysis, showing that a potent preparation of SFV-specific (H-2<sup>k</sup>) CTL was used. SFV-infected allogeneic P815 cells (H-2<sup>d</sup>), SFV-infected allogeneic EL4 cells (H-2<sup>b</sup>) and untreated Yac-1 cells were not killed by the SFV-specific CTL, hence killing was MHC restricted and there was no detectable natural killer cell activity in the effector population. C1300 cells (H-2<sup>a</sup>, D<sup>d</sup>K<sup>k</sup>) and the SFV-specific CTL (H-2<sup>k</sup>, D<sup>k</sup>K<sup>k</sup>) share an identical class I MHC H-2K region. That SFV-infected C1300 cells were not susceptible to lysis by SFV-specific

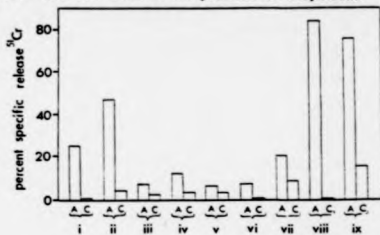
Figure 5.6

Effect of IFN- $\gamma$  on susceptibility of SFV-infected and  
SPISFV-treated C1300 cells to lysis by CVM/Na (H-2<sup>b</sup>) SFV-specific  
cytotoxic T-lymphocytes and anti-SFV serum in the presence of  
complement











(A) SFV-specific cytotoxic T-lymphocytes



(B) anti-SFV serum in the presence of complement



# Key

- (i),  C1300 cells (H-2D<sup>d<sub>k</sub>k<sub>k</sub></sup>) + IFN-γ + SFV
- (ii),  C1300 cells (H-2D<sup>d<sub>k</sub>k<sub>k</sub></sup>) + SFV
- (iii),  C1300 cells (H-2D<sup>d<sub>k</sub>k<sub>k</sub></sup>) + IFN-γ + BPLSFV
- (iv),  C1300 cells (H-2D<sup>d<sub>k</sub>k<sub>k</sub></sup>) + BPLSFV
- (v),  C1300 cells (H-2D<sup>d<sub>k</sub>k<sub>k</sub></sup>) + IFN-γ
- (vi),  C1300 cells (H-2D<sup>d<sub>k</sub>k<sub>k</sub></sup>) untreated
- (vii),  EL4 cells (H-2D<sup>b<sub>k</sub>k<sub>b</sub></sup>) + SFV
- (viii),  F815 cells (H-2D<sup>d<sub>k</sub>k<sub>d</sub></sup>) + SFV
- (ix),  L929 cells (H-2D<sup>k<sub>k</sub>k<sub>k</sub></sup>) + SFV
-  Yac-1 cells untreated

A, lysis by anti-SFV serum in presence of complement

C, lysis by complement alone

- (i) Cells were treated with 1000 u/ml IFN-γ or medium alone for 48 hours, prior to infection with SFV or treatment with an equivalent amount of BPLSFV. The susceptibility of the cells to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement were then determined, as described in methods section M.
- (ii) SFV-cytotoxic T-lymphocytes were derived from C3H/He mice (H-2<sup>k</sup>; D<sup>k<sub>k</sub>k<sub>k</sub></sup>) as described in methods section J, and were used in a 5 hour chromium release assay.

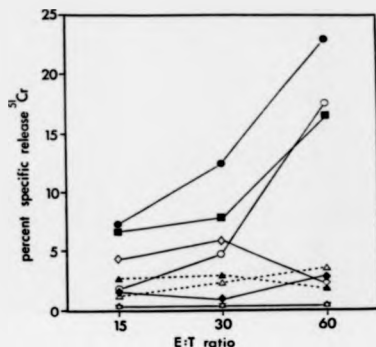
H-2<sup>k</sup>) CTL implies that the SFV-specific CTL are unable to recognise SFV antigen in association with H-2K<sup>b</sup> antigen on C1300 cells. C1300 cells are not inherently resistant to CTL lysis since they have previously been shown to be highly susceptible to alloreactive CTL lysis (see chapter 4 figure 4.29).

The effect of IFN- $\gamma$  treatment on the susceptibility of SFV-infected C1300 cells to lysis by both SFV-specific (H-2<sup>d</sup>) CTL (derived from Balb/c mice) and anti-SFV serum in the presence of complement were also investigated. However, in a series of experiments no detectable SFV-specific (H-2<sup>d</sup>, D<sup>d</sup>K<sup>d</sup>) CTL activity was observed when SFV-infected C1300 cells (H-2<sup>a</sup>, D<sup>d</sup>K<sup>k</sup>) and SFV-infected syngeneic P815 cells (H-2<sup>d</sup>, D<sup>d</sup>K<sup>d</sup>) were used as targets. SFV-infected C1300 cells and SFV-infected P815 cells were highly susceptible to lysis by anti-SFV serum in the presence of complement, indicating that the cells were displaying SFV antigen on the cell-surface (data not shown). Identical experiments were also set up using DBA/2 mice (H-2<sup>d</sup>) as the source of SFV-specific CTL, and again no CTL activity was observed (data not shown). Similarly, an experiment was set up using B10.A(5R) mice as the source of SFV-specific CTL. C1300 cells (H-2<sup>a</sup>, D<sup>d</sup>K<sup>k</sup>) and B10.A(5R) mice (H-2<sup>i5</sup>, D<sup>k</sup>K<sup>b</sup>) share an identical class I H-2D<sup>d</sup> antigen region. Yet again, no SFV-specific (H-2<sup>i5</sup>) CTL activity was observed when SFV-infected C1300 cells, SFV-infected P815 cells (H-2<sup>d</sup>, D<sup>d</sup>K<sup>d</sup>) and SFV-infected G26-24 cells (H-2<sup>b</sup>, D<sup>b</sup>K<sup>b</sup>) were used as targets. All target cells displayed SFV antigen on the cell-surface as determined by the susceptibility to lysis by anti-SFV serum in the presence of complement (data not shown). Finally, an experiment was set up using A/J mice as the source of SFV-specific CTL. C1300 cells (H-2<sup>a</sup>, D<sup>d</sup>K<sup>k</sup>) were derived from the A/J mouse, hence share identical class I MHC H-2D<sup>d</sup> and H-2K<sup>k</sup> regions. Figures 5.7a and 5.7b show the effect of IFN- $\gamma$  treatment on the susceptibility of SFV-infected C1300 cells to lysis by both SFV-specific (H-2<sup>a</sup>) CTL and anti-SFV serum in the presence of complement. It can be seen that non-IFN treated SFV-infected

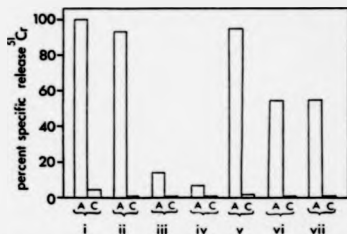
Figure 5.7

Effect of IFN- $\gamma$  on susceptibility of SFV-infected C100 cells to lysis by A/1 (H-2<sup>b</sup>) SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement

(A) SFV-specific cytotoxic T-lymphocytes



(B) anti-SFV serum in the presence of complement





# Key

- (i). (○—○) C1300 cells (H-2D<sup>d</sup>K<sup>k</sup>) + SFV
- (ii). (●—●) C1300 cells (H-2D<sup>d</sup>K<sup>k</sup>) + IFN-γ + SFV
- (iii). (Δ--Δ) C1300 cells (H-2D<sup>d</sup>K<sup>k</sup>) untreated
- (iv). (▲--▲) C1300 cells (H-2D<sup>d</sup>K<sup>k</sup>) + IFN-γ
- (v). (■—■) P815 cells (H-2D<sup>d</sup>K<sup>d</sup>) + SFV
- (vi). (◆—◆) L929 cells (H-2K<sup>k</sup>D<sup>k</sup>) + SFV
- (vii). (◇—◇) EL4 cells (H-2K<sup>b</sup>D<sup>b</sup>) + SFV
- (viii). (⊗—⊗) Yac-1 cells untreated

A. lysis by anti-SFV serum in presence of complement

C. lysis by complement alone

- (i) Cells were treated with 1000 u/ml IFN-γ or medium alone for 48 hours, prior to infection with SFV. The susceptibility of the cells to lysis by SFV-specific cytotoxic T-lymphocytes and anti-SFV serum in the presence of complement were then determined, as described in methods section M.
- (ii) SFV-cytotoxic T-lymphocytes were derived from A/J (H-2<sup>a</sup>; D<sup>d</sup>K<sup>k</sup>) as described in methods section J, and were used in a 5 hr chromium release assay.

C1300 cells were clearly susceptible to lysis by both SFV-specific (H-2<sup>a</sup>) CTL and anti-SFV serum in the presence of complement. IFN- $\gamma$  treated SFV-infected C1300 cells were markedly more susceptible to SFV-specific (H-2<sup>a</sup>) CTL lysis than non-IFN treated SFV-infected C1300 cells, despite a very slight reduction of SFV antigen expression on the cells as assessed by the slightly reduced susceptibility to lysis by anti-SFV serum in the presence of complement (and a marked reduction of SFV antigen expression on 32% of cells as assessed by indirect immunofluorescence staining, see chapter 4 figure 4.10). SFV-infected PB15 cells (H-2<sup>d</sup>, D<sup>d</sup><sub>K<sup>d</sup></sub>) were clearly susceptible to SFV-specific (H-2<sup>a</sup>, D<sup>d</sup><sub>K<sup>k</sup></sub>) CTL lysis. In contrast, SFV-infected allogeneic EL4 cells (H-2<sup>b</sup>, D<sup>b</sup><sub>K<sup>b</sup></sub>), SFV-infected L929 cells (H-2<sup>k</sup>, D<sup>k</sup><sub>K<sup>k</sup></sub>) and uninfected C1300 cells (either non-IFN treated or IFN- $\gamma$  treated) were not susceptible to SFV-specific (H-2<sup>a</sup>, D<sup>d</sup><sub>K<sup>k</sup></sub>) CTL lysis. Hence killing by the SFV-specific CTL was both specific and MHC restricted. Yac-1 cells were not killed showing that there was no detectable natural killer cell activity in the effector population.

### Discussion

The discussion has been split up into two sections due to the breadth of data presented in this chapter, (a) the effect of IFN treatment on the susceptibility of SFV-infected brain cells to SFV-specific CTL lysis, (b) genetic mapping of the SFV-specific CTL response.

#### (a) Effect of IFN treatment on the susceptibility of SFV-infected brain cells to SFV-specific CTL lysis

The data presented in this chapter show that non-IFN treated SFV-infected astrocytes displayed SFV antigen on the cell surface, as determined by the susceptibility to lysis by anti-SFV serum in the presence of complement (and as shown by indirect immunofluorescence staining, see chapter 4 figure 4.8), and were clearly susceptible to lysis by SFV-specific CTL. The fact that the lysis by SFV-specific CTL was found to be MHC restricted, and that at least some non-IFN treated astrocytes display class I MHC antigen on the cell surface (see chapter 4 figure 4.13) suggests that the non-IFN treated SFV-infected astrocytes were killed by class I MHC restricted SFV-specific CTL. Astrocytes treated with natural IFN- $\alpha\beta$ , prior to infection with SFV, showed a quantitatively similar level of susceptibility to SFV-specific CTL lysis as non-IFN treated SFV-infected astrocytes, despite a marked reduction of SFV antigen expression on the cells, as assessed by the reduced susceptibility to lysis by anti-SFV serum in the presence of complement (and by indirect immunofluorescence staining, see chapter 4 figure 4.7). Similarly, astrocytes treated with recombinant IFN- $\gamma$ , prior to infection with SFV, were found to be markedly more susceptible to SFV-specific CTL lysis than non-IFN treated SFV-infected astrocytes, again despite a marked reduction of SFV antigen expression on the cells, as

assessed by the reduced susceptibility to lysis by anti-SFV serum in the presence of complement (and by indirect immunofluorescence staining, see chapter 4 figure 4.8). Since virus-specific CTL recognise viral antigen in association with class I MHC antigen on the surface of an infected target cell, and both IFN- $\alpha\beta$  and IFN- $\gamma$  markedly increase class I MHC antigen expression on astrocytes (as assessed by indirect immunofluorescence staining and the increased susceptibility of IFN-treated astrocytes to alloreactive CTL lysis, see chapter 4 figures 4.11 to 4.13) the simplest explanation for these data is that although IFN- $\alpha\beta$  and IFN- $\gamma$  treatment markedly reduced SFV antigen expression on infected astrocytes, in the context of increased class I MHC antigen expression, IFN-treated SFV-infected astrocytes remained susceptible to lysis by SFV-specific CTL. Bearing in mind IFN- $\alpha\beta$  and IFN- $\gamma$  treatment inhibited SFV-replication in astrocytes (see chapter 4 figures 4.1 and 4.2) and essentially abolished SFV antigen display on infected cells, the question arises as to the source of SFV antigen which was recognised by the SFV-specific CTL. Experiments in which astrocytes treated with an equivalent amount of a  $\beta$ -propiolactone inactivated preparation of SFV (BPLSFV) were found to be susceptible to lysis by SFV-specific CTL, again despite expressing essentially no SFV antigen detectable by indirect immunofluorescence staining or lysis by anti-SFV serum in the presence of complement, suggest that the input SFV antigen with which the cells were challenged may be recognised by the SFV-specific CTL. Astrocytes treated with IFN- $\gamma$ , prior to treatment with BPLSFV, were also found to be markedly more susceptible to SFV-specific CTL lysis than non-IFN treated BPLSFV-treated astrocytes, and again this increase in susceptibility correlated with an increase in class I MHC antigen expression on the cells. That treatment with inactivated virus can sensitise cells for lysis by virus-specific CTL has also been reported previously in other systems. Thus, Schrader and Edelman (1977)

demonstrated that F813 mastocytoma cells treated with ultra-violet inactivated sendai virus were susceptible to lysis by sendai-virus specific CTL, and Yewdell and co-workers showed that L929 fibroblast cells treated with ultra-violet inactivated influenza virus (strain PR8, which was also heated at 55°C to inactivate viral neuraminidase activity and hence prevented the elution of virus from cellular sialic acid receptors) were lysed by influenza-virus specific CTL (Yewdell *et al.*, 1988b; Hosaka *et al.*, 1988). Since it is currently believed that virus-specific CTL recognise viral antigen peptide fragments which have bound to class I MHC antigen on the surface of a target cell (Townsend *et al.*, 1986; Bjorkman *et al.*, 1987a, 1987b; Bastin *et al.*, 1987) the observation in the present study that astrocytes treated with BPLSFV were susceptible to SFV-specific CTL lysis does at least suggest that the astrocytes were able to process SFV antigen into a form which could be recognised by the SFV-specific CTL. The ability of astrocytes to process antigen has not yet been formally demonstrated although Takiguchi and Frelinger (1986) showed that IFN- $\gamma$  treated astrocytes presented keyhole limpet hemocyanin (KLH) to the class II MHC restricted KLH-specific T-cell hybridoma SKK-43.10, which only recognises processed KLH antigen. That IFN- $\gamma$  treated SFV-infected astrocytes and BPLSFV-treated astrocytes (both non-IFN treated and IFN- $\gamma$  treated) were clearly susceptible to SFV-specific CTL lysis despite expressing essentially no detectable SFV antigen on the cell-surface, as assessed by the susceptibility to lysis by anti-SFV serum in the presence of complement and by indirect immunofluorescence staining, may be a reflection of the fact that T-cells and antibody recognise different forms of antigen. Thus, T-cells recognise linear viral peptide fragments between eight and twelve amino-acids long, whereas antibodies usually recognise sterically exposed non-linear viral epitopes involving approximately fifteen amino-acid contacts (Rothbard, 1987). Hence, if astrocytes do

indeed process SFV antigen into short peptide fragments, these will be recognised by SFV-specific T-cells but not by SFV-specific antibody. An alternative explanation is that T-cells are more sensitive than antibody in detecting viral antigen on the surface of cells.

It should be noted that a number of studies have recently reported the isolation of class II MHC restricted antigen-specific CTL clones (Morrison *et al.*, 1986, 1988; Jonas *et al.*, 1987). Class II MHC restricted CTL however constitute only a minor proportion of the total CTL population, the majority being class I MHC restricted (Braakman *et al.*, 1987). Since the SFV-specific T-cells used in the present studies were a mixed population, it is possible that some IFN- $\gamma$  treated SFV-infected astrocytes and IFN- $\gamma$  treated BPLSFV-treated astrocytes (which express class II MHC antigen on the cell surface, see chapter 4 figure 4.16) were killed by class II MHC restricted SFV-specific CTL. It does at least seem unlikely that non-IFN treated and IFN- $\alpha\beta$  treated SFV-infected astrocytes were killed by class II MHC restricted SFV-specific CTL, since these cells do not express any detectable class II MHC antigen on the cell-surface (see chapter 4 figure 4.17). Recently, Sun and Wekerle (1986) have shown that IFN- $\gamma$  treated astrocytes treated with myelin basic protein (MBP) were susceptible to lysis by a class II MHC restricted MBP-specific T-cell line. A future experimental approach which could be used to determine whether any IFN- $\gamma$  treated SFV-infected astrocytes were killed by class II MHC restricted SFV-specific CTL, would be to add anti-class II MHC antigen antibodies to co-cultures of astrocytes with SFV-specific CTL, during the cytotoxicity assay. A comparison of the levels of SFV-specific CTL lysis observed in the presence and absence of the anti-class II MHC antigen antibodies could then be made, and any reduction in the level of lysis assigned to class II MHC restricted SFV-specific cytotoxicity.

Since the astrocyte cultures were at least 90% GFAP<sup>+</sup> astrocyte in composition, and at least 86% of SFV-infected cells displayed SFV antigen on the cell-surface (see chapter 3), the observation that upto 87% SFV-specific CTL lysis occurred does at least indicate that astrocytes were indeed killed by the SFV-specific CTL. Whether the uncharacterised cells present (e.g. oligodendrocytes, neurones and microglial cells) were also killed by the SFV-specific CTL remains to be determined.

The effect of SFV-infection and BPLSFV-treatment on class I and class II MHC antigen expression by astrocytes were also investigated, since it was possible that infection with SFV or treatment with BPLSFV modulated the expression of MHC antigen on astrocytes during the cytotoxicity assays, and hence modulated the susceptibility of the cells to SFV-specific CTL lysis. The data presented show that SFV-infection of astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) and BPLSFV-treatment of non-IFN treated astrocytes had no effect on class I MHC H-2D<sup>k</sup>k<sup>k</sup> antigen expression. In contrast, BPLSFV-treatment of IFN- $\alpha\beta$  treated and IFN- $\gamma$  treated astrocytes very slightly reduced class I MHC H-2D<sup>k</sup>k<sup>k</sup> antigen expression (on 12% and 8% of cells, respectively) indicating that BPLSFV-treatment may have also slightly reduced the susceptibility of these cells to class I MHC restricted SFV-specific CTL lysis. SFV-infection and BPLSFV-treatment had no effect on class II MHC H-2A<sup>k</sup> antigen expression by non-IFN treated and IFN- $\alpha\beta$  treated astrocytes, but in contrast very slightly reduced H-2A<sup>k</sup> antigen expression on some IFN- $\gamma$  treated astrocytes (on 5% and 10% of cells for SFV-infection and BPLSFV-treatment [at 10 hours post-treatment], respectively) again indicating that SFV-infection and BPLSFV-treatment may have also slightly reduced the susceptibility of these cells to class II MHC restricted SFV-specific CTL lysis. Since BPLSFV-treatment was found to

reduce class II MHC H-2A<sup>k</sup> antigen expression on 10% of IFN- $\gamma$  treated astrocytes when stained at 10 hours post-infection, IFN- $\gamma$  treated astrocytes were also treated with BPLSFV for 4 days in order to determine whether a greater effect on H-2A<sup>k</sup> antigen expression would be observed. The data presented show that 4-day BPLSFV-treatment of IFN- $\gamma$  treated astrocytes had no effect on H-2A<sup>k</sup> antigen expression. Taken together these observations do at least indicate that the slight reduction of H-2A<sup>k</sup> antigen expression observed at 10 hours post-treatment may have only been due to a short-term disturbance of the cell-membrane which was brought about by <sup>a</sup>treatment of the IFN- $\gamma$  treated astrocytes with BPLSFV.

The data presented in this chapter also show that SFV-infected G26-24 oligodendrogloma cells and SFV-infected C1300 neuroblastoma cells displayed SFV antigen on the cell-surface, as determined by the susceptibility to lysis by anti-SFV serum in the presence of complement (and as shown by indirect immunofluorescence staining, see chapter 4 figures 4.9 and 4.10), and were susceptible to lysis by SFV-specific CTL (derived from C57BL/6 [H-2<sup>b</sup>] mice and A/J [H-2<sup>a</sup>] mice for G26-24 cells and C1300 cells, respectively). As was found with astrocytes, IFN- $\gamma$  treatment of G26-24 cells and C1300 cells, prior to infection with SFV, markedly increased the susceptibility of the cells to SFV-specific CTL lysis despite a reduction of SFV antigen display on the cells, as assessed by the reduced susceptibility to lysis by anti-SFV serum in the presence of complement (which was only very slight for C1300 cells) and by indirect immunofluorescence staining (see chapter 4 figures 4.9 and 4.10). The increase in susceptibility to SFV-specific CTL lysis correlated with an increase in class I MHC antigen expression (and class II MHC antigen expression for G26-24 cells) on the cells, as assessed by indirect immunofluorescence staining and the increased susceptibility of IFN-treated



cells to alloreactive CTL lysis (see chapter 4 figures 4.18 to 4.29) again indicating that although IFN- $\gamma$  treatment reduced SFV antigen display on (at least some) infected cells, in the context of increased MHC antigen expression IFN- $\gamma$  treated SFV-infected G26-24 cells and IFN- $\gamma$  treated SFV-infected C1300 cells remained susceptible to lysis by SFV-specific CTL. BPLSFV-treated non-IFN treated G26-24 cells were not killed by SFV-specific CTL. In contrast, IFN- $\gamma$  treated <sup>BPLSFV-treated</sup> G26-24 cells were highly susceptible to SFV-specific CTL lysis, indicating that the input SFV antigen with which the cells were challenged may be recognised by SFV-specific CTL, but only when MHC antigen expression on G26-24 cells is increased by treatment with IFN- $\gamma$ . Since at least some non-IFN treated G26-24 cells and non-IFN treated astrocytes display class I MHC antigen on the cell-surface (see chapter 4) the observations that BPLSFV-treated non-IFN treated astrocytes were highly susceptible to lysis by SFV-specific CTL and that BPLSFV-treated non-IFN treated G26-24 cells were not susceptible to lysis by SFV-specific CTL, do at least suggest that the astrocytes may have been more efficient at processing the input SFV antigen for recognition by SFV-specific CTL. Whether the observations that SFV-infected G26-24 oligodendroglioma cells and SFV-infected neuroblastoma cells were susceptible to lysis by SFV-specific CTL reflect the situation with normal oligodendrocytes and neurons, remains to be determined.

Blackman and Morris (1985) have also shown that RDM4 lymphoblastoid cells and C3H10T $\frac{1}{2}$  fibroblast cells treated with natural IFN- $\alpha\beta$  or natural IFN- $\gamma$ , prior to infection with SFV, remained susceptible to lysis by SFV-specific CTL despite a reduction in SFV antigen display on the cells (as assessed by the reduced susceptibility to lysis by anti-SFV serum in the presence of complement). Furthermore, natural IFN- $\alpha\beta$  and natural IFN- $\gamma$  treatment were found to increase class I MHC antigen expression on both RDM4 and C3H10T $\frac{1}{2}$

cells, as assessed by the increased susceptibility to alloreactive CTL lysis. This effect does not appear to be restricted to the SPV system since similar observations have also been reported in other virus-systems. Thus, Yewdell and co-workers demonstrated that treatment of adenovirus-12 transformed (Ad-12) cells with IFN- $\gamma$  (not specified whether natural or recombinant), prior to infection with influenza virus, resulted in a decreased expression of influenza virus genes yet the cells were markedly more susceptible to lysis by influenza-specific CTL. The increase in susceptibility to lysis by influenza-specific CTL also correlated with an increase in class I MHC antigen expression on the cells (Yewdell *et al.*, 1988a; Eger *et al.*, 1983). Similarly, King and co-workers demonstrated that treatment of Balb/c mouse embryo fibroblasts with a supernatant harvested from concanavalin-A stimulated splenocyte cultures (which contained IFN- $\gamma$ ), prior to infection with murine cytomegalovirus (MCMV), increased the susceptibility of the cells to lysis by MCMV-specific CTL. Again, the increased susceptibility to lysis by MCMV-specific CTL was found to correlate with an increase in class I MHC antigen expression on the cells, although the effects of IFN- $\gamma$  treatment on MCMV replication and antigen display were not investigated in this study (King *et al.*, 1985). A number of studies have also shown that where cells are infected with virus, prior to IFN treatment, viral antigen display on the cells may not always be reduced by IFN treatment, and again in such situations it was found that the susceptibility of IFN-treated targets to lysis by virus-specific CTL was increased. For example, Bukowski and Welsh (1985) demonstrated that natural IFN- $\alpha\beta$  and natural IFN- $\gamma$  treatment of low passage murine embryonic fibroblasts, infected with lymphocytic choriomeningitis virus (LCMV), had no effect on the level of LCMV antigen expressed on the surface of the cells, but increased the susceptibility of the cells to LCMV-specific CTL lysis. Once again, the increase in susceptibility to lysis was found to

correlate with an increase in class I MHC antigen expression on the cells. Subsequent studies by these workers (Bukowski and Welsh, 1986) also demonstrated that natural IFN- $\beta$  treatment of LCMV-infected fibroblasts derived from either the brain, heart, liver or skin, rendered the cells more susceptible to lysis by LCMV-specific CTL, and that the increase in susceptibility correlated with an increase in class I MHC antigen expression on the cells. Similarly, Blackman and Morris (1985) and Flyer *et al.* (1985) showed that natural IFN- $\alpha\beta$  and natural IFN- $\gamma$  treatment of fibroblasts, chronically infected with the retroviruses murine sarcoma virus/murine leukemia virus (MSV/MLV) (Kirsten or Moloney strains), increased both class I MHC antigen expression on the cells and the susceptibility of the cells to lysis by virus-specific CTL.

The general conclusion from each of the experimental systems so far discussed is that whenever the level of class I MHC antigen expression on a virus-infected cell is increased by treatment with IFN, there results an increased susceptibility of the cell to lysis by virus-specific CTL. However, it is important to note that some cells may constitutively express very high levels of class I MHC antigen and in such cases IFN treatment may not increase the level of class I MHC antigen expression nor the susceptibility of the cells to CTL lysis. For example, Bukowski and Welsh (1985) demonstrated that natural IFN- $\beta$  treatment of the continuous cell line MC57G, which expressed high levels of class I MHC antigen, and which had been previously infected by either LCMV or vaccinia virus, did not increase the level of class I MHC antigen expressed by the cells and did not increase the already high susceptibility of these cells to lysis by virus-specific CTL. It is also important to note that in cases where IFN treatment was found to increase the susceptibility of virus-infected cells to lysis by virus-specific CTL, the increase in susceptibility may not have

been due solely to the IFN-induced increase in class I MHC antigen expression, since other cell-surface molecules may have played a role in the interaction between the virus-specific CTL and target cell. For example, it is now clear that cell-adhesion molecules enhance the efficiency of specific receptor-dependant lymphocyte-target cell interactions (Springer *et al.*, 1987). To date, two receptor-ligand pairs involved in these interactions have been defined. Thus, the CD2 receptor interacts with the ligand lymphocyte function associated antigen-3 (LFA-3), whilst the LFA-1 receptor interacts with the ligand intercellular adhesion molecule-1 (ICAM-1). The CD2 and LFA-1 receptors are expressed on the surface of T-cells (and with the exception of some macrophages on all other leukocytes for the case of LFA-1, and on thymocytes and large granular lymphocytes for the case of CD2) whereas LFA-3 and ICAM-1 are widely distributed on the surface of a variety of cell-types (Dustin *et al.*, 1986; Springer *et al.*, 1987). A number of studies have recently shown that IFN- $\gamma$  treatment increases ICAM-1 expression on a variety of cell-types (reviewed by Dustin *et al.*, 1988) indicating that the IFN- $\gamma$  treated cells may also have an increased ability to interact with T-cells (which express the LFA-1 receptor). Of particular relevance to the data presented in this chapter are the unpublished observations of Frohman (research laboratory location not stated) who has demonstrated that IFN- $\gamma$  treatment of astrocytes increased ICAM-1 expression (reported by Dustin *et al.*, 1988). It does thereby seem a strong possibility that the increased susceptibility of IFN- $\gamma$  treated SFV-infected astrocytes to lysis by SFV-specific CTL, may have been a reflection of both increased class I (and class II) MHC antigen expression and increased ICAM-1 expression on the cells. Whether IFN- $\alpha\beta$  treatment also increases ICAM-1 (or LFA-3) expression on astrocytes remains to be determined. Similarly, whether IFN- $\alpha\beta$  or IFN- $\gamma$  treatment increase ICAM-1 (or LFA-3) expression on C26-24 cells and C1300 cells, remains to be

determined.

(b) Genetic mapping of the SFV-specific CTL response

C1300 neuroblastoma cells were derived from the recombinant A/J mouse which encodes the class I MHC region alleles H-2D<sup>d</sup> and H-2K<sup>k</sup>. By using SFV-specific CTL derived from inbred strains of mice which shared an identical H-2D<sup>d</sup> or H-2K<sup>k</sup> region, it was possible independently to study the SFV-specific CTL responses to H-2D<sup>d</sup> and H-2K<sup>k</sup> region products on SFV-infected C1300 cells. The data presented show that SFV-infected C1300 cells (D<sup>d</sup>K<sup>k</sup>) were not susceptible to lysis by SFV-specific CTL derived from C3H/He mice (D<sup>k</sup>K<sup>k</sup>) despite sharing an identical H-2K<sup>k</sup> region. In contrast, SFV-infected syngeneic L929 cells (D<sup>k</sup>K<sup>k</sup>) and SFV-infected syngeneic astrocytes (D<sup>k</sup>K<sup>k</sup>) were clearly susceptible to lysis by SFV-specific (D<sup>k</sup>K<sup>k</sup>) CTL. The C1300 cell H-2K<sup>k</sup> region product (H-2K<sup>k</sup> antigen) was functional since it was previously shown to act as a target for recognition by alloreactive CTL (see chapter 4 figure 4.29). Taken together these data indicate that the SFV-specific (D<sup>k</sup>K<sup>k</sup>) CTL were unable to recognise SFV antigen in association with H-2K<sup>k</sup> antigen, and suggest that the SFV-specific CTL response was associated with the H-2D region products of the H-2<sup>k</sup> haplotype. Similar observations have also been reported previously by Mullbacher and Blanden (1978) who demonstrated that SFV-specific CTL derived from CBA mice (D<sup>k</sup>K<sup>k</sup>) only lysed SFV-infected macrophage targets which shared an identical H-2D<sup>k</sup> region, and did not lyse targets which shared an identical H-2K<sup>k</sup> region. The data presented in this chapter also show that SFV-infected L929 cells (D<sup>k</sup>K<sup>k</sup>) were not susceptible to lysis by SFV-specific (D<sup>d</sup>K<sup>k</sup>) CTL (derived from A/J mice) despite sharing an identical H-2K<sup>k</sup> region. Again, this observation suggests that the SFV-specific CTL were unable to recognise SFV antigen in association with H-2K<sup>k</sup> antigen. In contrast, SFV-infected C1300

cells ( $D^d_k$ ) were clearly susceptible to lysis by syngeneic SFV-specific ( $D^d_k$ ) CTL. Since SFV-specific CTL do not recognise SFV antigen in association with H-2K<sup>k</sup> antigen, the SFV-specific ( $D^d_k$ ) CTL must have recognised SFV antigen on C1300 cells in association with H-2D<sup>d</sup> region products. The H-2D region contains the genes which encode both the H-2D and H-2L antigens (Biddison *et al.*, 1978; Male *et al.*, 1987). It is thereby possible that the SFV-specific ( $D^d_k$ ) CTL may have recognised SFV antigen on C1300 cells in association with H-2D antigen and/or H-2L antigen. To date, H-2L restricted virus-specific CTL responses have only been demonstrated in a small number of virus systems (an example being the influenza virus system: Biddison *et al.*, 1978). A future experimental approach which could be used to determine whether the SFV-specific ( $D^d_k$ ) CTL recognised SFV antigen on C1300 cells in association with H-2D antigen or H-2L antigen, would be to add anti-H-2D serum or anti-H-2L serum to the co-cultures of SFV-infected C1300 cells with SFV-specific ( $D^d_k$ ) CTL during subsequent cytotoxicity assays and monitor the effect on the level of lysis observed. Any reduction in the level of SFV-specific CTL lysis observed in the presence of anti-H-2D serum or anti-H-2L serum could then be assigned to H-2D antigen and H-2L antigen restricted SFV-specific CTL activity, respectively. As mentioned previously, a number of studies have recently demonstrated class II MHC restricted antigen-specific CTL activity. The possibility that the SFV-specific CTL responses actually mapped to the class II MHC region cannot be excluded. This does however at least seem unlikely since class II MHC restricted CTL only constitute a minor proportion of the total CTL population and C1300 cells (both non-IFN treated and IFN- $\gamma$  treated) do not display any detectable class II MHC antigen on the cell surface (see chapter 4 figure 4.30). The data presented in this chapter also show that SFV-infected G26-24 oligodendrogloma cells ( $D^b_k$ ) were susceptible to lysis by syngeneic SFV-

specific ( $D^bK^b$ ) CTL (derived from C57 BL/6 mice). It is not possible from the data obtained to deduce whether the SFV-specific ( $D^bK^b$ ) CTL recognised SFV antigen in association with H-2D or H-2K region products on G26-24 cells. That the SFV-specific CTL response was associated with the H-2D region products of the H-2<sup>k</sup> haplotype does not mean that the SFV-specific CTL response is also associated with the H-2D region of the H-2<sup>b</sup> haplotype, since responses also vary with haplotype. For example, Kesson and co-workers have demonstrated that the West Nile virus (WNV) specific CTL response was associated with the K region of the H-2<sup>k</sup> haplotype and both the K and D regions of the H-2<sup>d</sup> haplotype (Kesson *et al.*, 1988). The observation in the present study that a clear SFV-specific CTL activity was generated in C57 BL/6 mice (H-2<sup>b</sup>) clearly contrasts with the results of Mullbacher and Blanden (1978) who were unable to detect any SFV-specific CTL activity in this system. The reason for this discrepancy in results is not clear since both studies used a population of SFV-specific CTL which were prepared by an essentially identical protocol. In the present study, no detectable SFV-specific CTL activity was generated in either the Balb/c (H-2<sup>d</sup>) or DBA/2 (H-2<sup>d</sup>) mouse system. These observations are consistent with the results of Mullbacher and Blanden (1978) who also failed to demonstrate any clear SFV-specific CTL activity in the Balb/c mouse system. Since Balb/c mice (H-2<sup>d</sup>,  $D^dK^d$ ) and A/J mice (H-2<sup>a</sup>,  $D^dK^k$ ) both encode an identical class I MHC H-2D<sup>d</sup> region, the question arises as to why a H-2D<sup>d</sup>-restricted SFV-specific CTL response was demonstrated in the A/J mouse system but not in the Balb/c mouse system. A possible explanation may be that the generation of H-2D<sup>d</sup>-restricted SFV-specific CTL was controlled by class II MHC I-region genes, and the A/J mouse encodes 'responder' I<sup>k</sup> region genes whereas the Balb/c mouse encodes 'non-responder' I<sup>d</sup> region genes. It is also interesting to note that a SFV-specific CTL response was demonstrated in the C3H/He mouse system, which encodes I<sup>k</sup> region genes.

The observation that a SFV-specific CTL response was generated in the C57 BL/6 ( $D^b I^b K^b$ ) mouse system also suggests that the C57 BL/6 mouse encodes responder  $I^b$  region genes. The B10.A(5R) mouse ( $H-2^{15}$ ,  $K^b I^{bk} D^d$ ) has a recombinant I-region composed of genes derived from both  $H-2^b$  and  $H-2^k$  mice, and encodes  $H-2D^b$  region genes. The observation in the present study that no detectable  $H-2D^d$ -restricted SFV-specific CTL response was generated in the B10.A(5R) mouse system, indicates that the recombinant  $I^{bk}$  region (which was derived from two responder I- regions) was a non-responder region.



Effect of IFN on the ability of brain cells to present SFV antigen to  
SFV-specific T-cells as assessed by proliferation and the release of IFN- $\gamma$

Introduction

It is now well established that an essential step in the induction of an immune response to a protein antigen is the activation of T-helper cells. As discussed in detail in chapter 1, this activation cannot be induced by free antigen but only by antigen presented by an accessory cell which displays class II MHC antigen on the cell surface. Since the expression of class II MHC antigen is essential for antigen presentation (and hence the development of helper and cytotoxic T-cells, as well as antibody formation) it follows that any modulation of class II MHC antigen expression may significantly influence the immune response.

In chapter 4, data were presented which show that IFN- $\gamma$  treatment induced class II MHC antigen expression on astrocytes. In this chapter the effect of IFN- $\gamma$  treatment on the ability of astrocytes to present SFV antigen to SFV-specific T-cells is investigated, via T-cell proliferation assay. Since virus-specific CTL and T-helper cells release IFN- $\gamma$  only on appropriate recognition of viral antigen (Morris et al., 1982; Cunningham et al., 1985) the release of IFN- $\gamma$  by virus-specific T-cells can be used as a marker for T-cell recognition of antigen. The effect of IFN- $\alpha\beta$  and IFN- $\gamma$  on the ability of astrocytes and G26-24 cells to stimulate SFV-specific T-cell release of IFN- $\gamma$  is thereby also investigated.

## Results

### Effect of IFN on the ability of astrocytes to present SFV antigen to SFV-specific T-cells

The effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  treatment on the ability of BPLSFV-treated astrocytes to present SFV antigen to SFV-specific T-cells was assessed via proliferation assay as described in methods section M. Astrocytes (untreated, IFN- $\alpha\beta$  treated and IFN- $\gamma$  treated) were first treated with a  $\beta$ -propiolactone inactivated preparation of SFV (BPLSFV) and then with mitomycin C (to prevent their subsequent proliferation) after which the cells were co-cultured with SFV-specific T-cells and the incorporation of [ $^3$ H]thymidine monitored over 24 hour periods upto day 7 post-co-culture. Indomethacin (a potent inhibitor of prostaglandin synthesis: Fontana *et al.*, 1986) was also added to the co-cultures to a final concentration of 10  $\mu$ g/ml, since astrocytes have previously been shown to produce prostaglandin E (Fontana *et al.*, 1982) which is known to suppress T-cell mediated immune responses (Male *et al.*, 1987). In a series of experiments it was found that BPLSFV-treated astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) did not stimulate SFV-specific T-cell proliferation since the amounts of [ $^3$ H]thymidine incorporated into co-cultures of BPLSFV-treated astrocytes with SFV-specific T-cells and into co-cultures of non-BPLSFV treated astrocytes with SFV-specific T-cells were essentially identical (data not shown). However, no conclusions regarding the ability of astrocytes to present SFV antigen to SFV-specific T-cells can be made from these studies since BPLSFV-treated syngeneic C3H/He splenocytes (used as an experimental positive control) also did not stimulate any detectable SFV-specific T-cell proliferation (data not shown). Antigen-specific T-cell proliferation requires the presence of interleukin-2 (IL-2, T-cell

growth factor: Balkwill, 1987). A further series of experiments were thereby set-up in which either recombinant IL-2 (at a final concentration of 10 u/ml) or a concanavalin-A stimulated rat splenocyte supernatant ('conditioned medium' which contains IL-2, used at a 1:10 dilution) were added to the co-cultures of BPLSFV-treated astrocytes with SFV-specific T-cells, in an attempt to assist any T-cell proliferation. However, again no SFV-specific T-cell proliferation was observed (data not shown). The failure to detect T-cell proliferation does not appear to be due to technical problems since IFN- $\gamma$  treated astrocytes were previously found to stimulate the proliferation of an alloreactive T-cell line (see chapter 4, table 4.1).

Effect of IFNs on the ability of BPLSFV-treated astrocytes and BPLSFV-treated G26-24 cells to stimulate SFV-specific T-cell release of IFN- $\gamma$

The effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  treatment on the ability of BPLSFV-treated astrocytes to stimulate SFV-specific T-cell release of IFN- $\gamma$  was investigated as described in methods section N.

The data in table 6.1 show the titres of IFN activity detected in supernatants harvested from co-cultures of astrocytes with SFV-specific T-cells, and from cultures of astrocytes alone. It can be seen that astrocytes alone released small amounts of IFN when treated with BPLSFV (between 3 and  $32 \pm 6$  u/ml, see data in column headed 'supernatants from cultures of astrocytes alone'). The IFN type appears to be IFN- $\alpha\beta$  since activity was neutralised by anti-IFN- $\alpha\beta$  serum but not by a monoclonal antibody to IFN- $\gamma$ . Anti-IFN- $\alpha\beta$  serum did not neutralise any IFN- $\gamma$  activity in a control experiment (data not shown). Non-BPLSFV-treated astrocytes

Table 6.1

Effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on ability of BPLSFV-treated astrocytes to stimulate SFV-specific T-cell release of IFN- $\gamma$

Astrocyte treatment	Supernatants from co-culture of astrocytes with SFV T-cells			Supernatants from cultures of astrocytes alone		
	Alone	+ Anti-IFN- $\alpha\beta$	+ Anti-IFN- $\gamma$	Alone	+ Anti-IFN- $\alpha\beta$	+ Anti-IFN- $\gamma$
IFN $\gamma$ + BPLSFV	131 $\pm$ 14 <sup>+</sup>	82 $\pm$ 6	20 $\pm$ 9	32 $\pm$ 6	<3	32 $\pm$ 7
BPLSFV alone	20 $\pm$ 1	10 $\pm$ 1	<3	3	<3	3
IFN $\alpha\beta$ + BPLSFV	72 $\pm$ 14	57 $\pm$ 11	<3	10 $\pm$ 7	<3	3
IFN $\gamma$ alone	<3	N.D. <sup>*</sup>	N.D.	<3	N.D.	N.D.
IFN $\alpha\beta$ alone	<3	N.D.	N.D.	<3	N.D.	N.D.
Untreated	<3	N.D.	N.D.	<3	N.D.	N.D.

<sup>+</sup>; titres refer to mean of six replicate titrations  $\pm$  S.D. units/ml IFN activity.

<sup>\*</sup>; N.D. not done.

- (i) Experimental protocol as described in methods section N.
- (ii) Cells treated with 1000 u/ml IFN- $\alpha\beta$ , 100 u/ml IFN- $\gamma$  or medium alone.
- (iii) Supernatants were harvested from cultures of astrocytes, treated as indicated, in the presence and absence of SFV-specific T-cells (derived from C3H/He mice). IFN activity was assessed and characterized as described in methods section F.

did not release any detectable IFN. Supernatants harvested from co-cultures of non-BPLSFV treated astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) with SFV-specific T-cells also contained no detectable IFN activity. In contrast, supernatants harvested from co-cultures of non-IFN treated BPLSFV-treated astrocytes with SFV-specific T-cells contained small amounts of IFN ( $20 \pm 1$  u/ml). The IFN type appears to be IFN- $\gamma$  since activity was significantly neutralised by the monoclonal antibody to IFN- $\gamma$  (from  $20 \pm 1$  u/ml to  $< 3$  u/ml, Students t-test  $p < 0.001$ ). The anti-IFN- $\gamma$  monoclonal antibody did not neutralise any IFN- $\alpha\beta$  activity in a control experiment (data not shown). That anti-IFN- $\alpha\beta$  serum also apparently significantly neutralised some activity (from  $20 \pm 1$  u/ml to 10 u/ml, Students t-test  $p < 0.001$ ) suggests that small amounts of IFN- $\alpha\beta$  were also present, and at a level which corresponds to the background production of IFN- $\alpha\beta$  due to BPLSFV-treatment of the astrocytes (3 u/ml). The observation that the anti-IFN- $\gamma$  monoclonal antibody specifically neutralised all IFN activity present in this supernatant, yet anti-IFN- $\alpha\beta$  serum also specifically neutralised some activity, probably reflects the fact that the IFN activities were titrated in a biological assay which inherently involve a background level of error. Supernatants harvested from co-cultures of IFN- $\alpha\beta$  treated BPLSFV-treated astrocytes with SFV-specific T-cells were found to contain significantly (Students t-test  $p < 0.001$ ) higher titres of IFN activity ( $72 \pm 14$  u/ml) than the supernatants harvested from co-cultures of non-IFN treated BPLSFV-treated astrocytes with SFV-specific T-cells ( $20 \pm 1$  u/ml). The predominant IFN type present appears to be IFN- $\gamma$  since activity was neutralised by the monoclonal antibody to IFN- $\gamma$  (from  $72 \pm 14$  u/ml to  $< 3$  u/ml). Anti-IFN- $\alpha\beta$  serum also significantly neutralised some activity (from  $72 \pm 14$  u/ml to  $57 \pm 11$  u/ml, Students t-test  $p < 0.1$ ) indicating that small amounts of IFN- $\alpha\beta$  were also present, and at a level which corresponds to the background production of

IFN- $\alpha\beta$  due to BPLSFV treatment of the astrocytes ( $10 \pm 7$  u/ml). Supernatants harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated astrocytes with SFV-specific T-cells were also found to contain significantly (Students t-test  $p < 0.001$ ) higher titres of IFN activity ( $131 \pm 14$  u/ml) than the supernatants harvested from co-cultures of non-IFN treated BPLSFV-treated astrocytes with SFV-specific T-cells ( $20 \pm 1$  u/ml). The predominant IFN type present again appears to be IFN- $\gamma$  since most activity was neutralised by the monoclonal antibody to IFN- $\gamma$  (from  $131 \pm 14$  u/ml to  $20 \pm 9$  u/ml). Anti-IFN- $\alpha\beta$  also significantly neutralised some activity (from  $131 \pm 14$  u/ml to  $82 \pm 6$  u/ml, Students t-test  $p < 0.001$ ) again indicating that small amounts of IFN- $\alpha\beta$  were present, and at a level which corresponds to the background level of IFN- $\alpha\beta$  production due to BPLSFV-treatment of the astrocytes ( $32 \pm 6$  u/ml). The titres of IFN activity detected in supernatants harvested from co-cultures containing IFN- $\alpha\beta$  treated BPLSFV-treated astrocytes ( $72 \pm 14$  u/ml) and IFN- $\gamma$  treated BPLSFV-treated astrocytes ( $131 \pm 14$  u/ml) were also found to be significantly different (Students t-test  $p < 0.001$ ). The release of IFN- $\gamma$  was also found to be MHC restricted, since WEHI 3b cells (H-2<sup>d</sup>) treated in the same manner did not stimulate the SFV-specific T-cell release of IFN- $\gamma$  (data not shown).

The preparation of BPLSFV used in these studies was prepared from a stock of SFV grown in BHK cells (see methods section A). Since this preparation must also contain BHK cell proteins, it was possible that the T-cells were not releasing IFN- $\gamma$  in response to SFV antigen but in response to BHK cell antigen. To investigate this possibility astrocytes (untreated, IFN- $\alpha\beta$  treated and IFN- $\gamma$  treated) were treated with a BHK cell lysate (prepared in the same manner as for the production of an SFV stock, with the exception that SFV infection was omitted and cells were removed using glass beads)

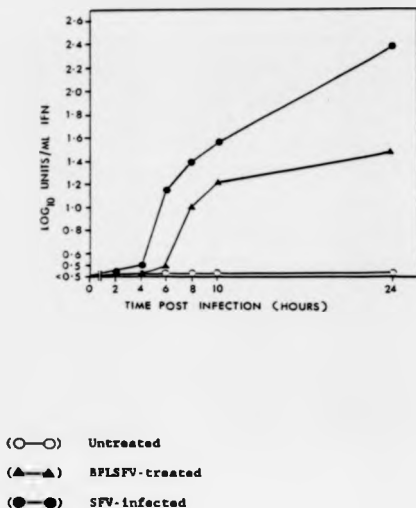
and the ability to stimulate SFV-specific T-cell release of IFN- $\gamma$  assessed. The results obtained clearly showed that BMK-cell-lysate treated astrocytes did not stimulate the SFV-specific T-cell release of IFN- $\gamma$  (data not shown). Hence, the release of IFN- $\gamma$  described in the above experiment must have been in response to SFV antigen.

Since astrocytes were found to release IFN- $\alpha\beta$  when treated with BPLSFV, a comparison was also made of the amount of IFN- $\alpha\beta$  released by astrocytes when treated with BPLSFV and infected with SFV. As shown in figure 6.1, SFV-infected astrocytes clearly released higher titres of IFN- $\alpha\beta$  than BPLSFV-treated astrocytes. Untreated astrocytes did not release any detectable IFN- $\alpha\beta$ .

The effect of the supernatants harvested from co-cultures of astrocytes with SFV-specific T-cells, and from cultures of astrocytes alone, on class II MHC H-2A<sup>k</sup> antigen expression by astrocytes was also investigated. H-2A<sup>k</sup> antigen expression was assessed by indirect immunofluorescence staining with quantification by flow cytometry. As shown in table 6.2, all supernatants harvested from cultures of astrocytes alone had no effect on H-2A<sup>k</sup> antigen expression by astrocytes, since the fluorescence of cells treated with these supernatants was essentially identical to background fluorescence. Supernatants harvested from co-cultures of non-BPLSFV-treated astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) with SFV-specific T-cells also had no effect on H-2A<sup>k</sup> antigen expression by astrocytes. In contrast, supernatants harvested from co-cultures of non-IFN treated BPLSFV-treated astrocytes with SFV-specific T-cells markedly increased H-2A<sup>k</sup> antigen expression on astrocytes, with a shift in mean fluorescence from 13 for untreated astrocytes to 40 for supernatant-treated astrocytes, and an increase in the percentage of cells

Figure 6.1

Production of IFN- $\alpha$  by SFV-infected and BPLSFV-treated astrocyte cultures



- (1) Astrocytes were mock-infected, treated with BPLSFV or infected with SFV as described in methods section M. At 1 hour post-infection monolayers were washed three times with fresh medium and 2 ml fresh medium replaced. Medium supernatants were then harvested at various times post-infection and dialysed twice against pH2 glycine buffer overnight followed by a final dialysis against PBS. IFN was then assayed as described in methods section F.



Table 6.2

Effect of supernatants harvested from co-cultures of astrocytes and SFV-specific T-cells on class II H-2A<sup>k</sup> antigen expression by astrocytes

Astrocyte Treatment	Fluorescence Intensity Distribution			
	Supernatants from co-cultures of astrocytes with SFV-T-cells		Supernatant from astrocytes alone	
	MN <sup>*</sup>	% <sup>+</sup>	MN	%
IFN $\gamma$ + BPLSFV	48	30	10	3
BPLSFV alone	40	26	14	7
IFN $\alpha$ + BPLSFV	53	33	14	6
IFN $\gamma$ alone	17	9	19	10
IFN $\alpha$ alone	18	10	14	6
Untreated	18	9	19	10

Background fluorescence (non-supernatant treated stained for H-2A<sup>k</sup> antigen)

; MN = 13, % = 5.

\* MN; mean fluorescence

<sup>+</sup> % : percentage of cells above 5% background fluorescence marker (non-supernatant treated, stained with an irrelevant antibody).

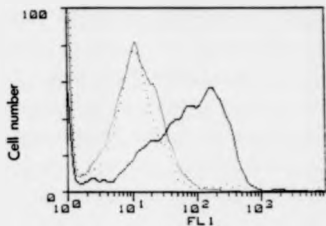
- (1) Supernatants were harvested from wells containing astrocytes treated as indicated, and cultured in the presence or absence of SFV-specific T-cells as described in methods section N. Supernatants were then diluted 1:4 prior to addition to astrocyte cultures for 48 hours. Class II (H-2A<sup>k</sup>) antigen was then stained by indirect immunofluorescence as described in methods section H.
- (11) 10000 cells analysed in each case using the FACS440 at the University of Birmingham, operated by Miss A Milner.

collected above the background fluorescence marker from 5% for untreated astrocytes to 26% for supernatant-treated astrocytes. Supernatants harvested from co-cultures of IFN- $\alpha\beta$  treated BPLSFV-treated astrocytes with SFV-specific T-cells increased H-2A<sup>k</sup> antigen expression on astrocytes to an even higher level, with a shift in mean fluorescence from 13 for untreated astrocytes to 53 for supernatant-treated astrocytes, and an increase in the percentage of cells collected above the background fluorescence marker from 5% for untreated astrocytes to 33% for supernatant treated astrocytes. Similarly, supernatants harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated astrocytes with SFV-specific T-cells increased H-2A<sup>k</sup> antigen expression on astrocytes to a high level, with a shift in mean fluorescence from 13 for untreated astrocytes to 48 for supernatant-treated astrocytes, and an increase in the percentage of cells collected above the background fluorescence marker from 5% for untreated astrocytes to 30% for supernatant-treated astrocytes.

The effect of anti-IFN- $\gamma$  monoclonal antibody on the ability of supernatant harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated astrocytes with SFV-specific T-cells to increase H-2A<sup>k</sup> antigen expression on astrocytes was also investigated. As shown in figure 6.2, the supernatant alone markedly increased H-2A<sup>k</sup> antigen expression on astrocytes, with an increase in the percentage of cells collected above the 5% background fluorescence marker from 7% for untreated astrocytes to 62% for supernatant treated astrocytes. The monoclonal antibody to IFN- $\gamma$  completely inhibited the ability of the co-culture supernatant to increase H-2A<sup>k</sup> antigen expression on astrocytes, with a decrease in the percentage of cells collected above the 5% background fluorescence marker from 62% for astrocytes treated with supernatant alone to 7% for astrocytes treated with supernatant and anti-IFN- $\gamma$  antibody. These observations suggest that IFN- $\gamma$  was the factor

Figure 6.2

Effect of anti-IFN- $\gamma$  antibody on ability of supernatant harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated astrocytes with SFV-T-cells to induce class II H-2A<sup>k</sup> antigen expression on astrocytes



**Key**

- solid line : co-culture supernatant alone
- spaced dots : co-culture supernatant + anti-IFN- $\gamma$  antibody
- close dots : untreated

FL1 : Fluorescence intensity distribution

- (1) Supernatants were harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated astrocytes and SFV-T-cells as described in methods section N. Supernatants were then diluted 1:4 prior to addition to astrocyte cultures for 48 hours in the presence or absence of a 1:100 dilution of H4-6A2 anti-IFN- $\gamma$  antibody ascites fluid. Class II (H-2A<sup>k</sup>) antigen was then stained by indirect immunofluorescence as described in methods section N.
- (11) 5000 cells analysed in each case.

present in the co-culture supernatant which increased H-2A<sup>b</sup> antigen expression on the astrocytes.

The effect of the supernatants on class I MHC H-2K<sup>b</sup> antigen expression by astrocytes was also investigated. As shown in table 6.3, all supernatants harvested from cultures of astrocytes alone (with the exception of IFN- $\gamma$  treated BPLSFV-treated astrocytes) had essentially no effect on H-2K<sup>b</sup> antigen expression by astrocytes, since the fluorescence of cells treated with these supernatants were essentially identical to background fluorescence. Supernatant harvested from cultures of IFN- $\gamma$  treated BPLSFV-treated astrocytes alone slightly increased H-2K<sup>b</sup> antigen expression on astrocytes, with an increase in mean fluorescence from 27 for untreated astrocytes to 44 for supernatant treated astrocytes, and an increase in the percentage of cells collected above the 5% background fluorescence marker from 18% for untreated astrocytes to 32% for supernatant-treated astrocytes. The ability of this supernatant to increase H-2K<sup>b</sup> antigen expression on astrocytes is probably due to the presence of small amounts of IFN- $\alpha\beta$ , produced by the astrocytes when treated with BPLSFV (see table 6.1). Supernatants harvested from co-cultures of non-BPLSFV-treated astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) with SFV-specific T-cells also slightly increased H-2K<sup>b</sup> antigen expression on astrocytes, with an increase in mean fluorescence from 27 for untreated astrocytes to around 44 for supernatant-treated astrocytes, and an increase in the percentage of cells collected above the 5% background fluorescence marker from 18% for untreated astrocytes to around 31% for supernatant-treated astrocytes. The ability of these supernatants to increase H-2K<sup>b</sup> antigen expression on astrocytes existed despite the absence of detectable IFN (see table 6.1). The most likely explanation for these data is that the SFV-specific T-cells released very small amounts of IFN- $\gamma$  which was

Table 6.3

Effect of supernatants harvested from co-cultures of astrocytes with SFV-specific T-cells on class I H-2K<sup>k</sup> antigen expression by astrocytes

Astrocyte treatment	Fluorescence intensity distribution			
	Supernatants from co-cultures of astrocytes with SFV-T-cells		Supernatant from astrocytes alone	
	MN <sup>+</sup>	% <sup>+</sup>	MN	%
IFN- $\gamma$ + BFLSFV	77	48	44	32
BFLSFV alone	84	54	32	22
IFN- $\alpha\beta$ + BFLSFV	91	58	26	14
IFN- $\gamma$ alone	47	34	28	17
IFN- $\alpha\beta$ alone	44	31	33	23
Untreated	46	31	29	19

Background fluorescence (non-supernatant treated stained for H-2K<sup>k</sup> antigen): MN = 27, % = 18.

\* MN: Mean fluorescence

\* %: percentage of cells above 5% background fluorescence marker (non-supernatant treated, stained with an irrelevant antibody).

- (i) Figure legend as for Table 6.2, except cells were stained for H-2K<sup>k</sup> antigen by indirect immunofluorescence.
- (ii) 10 000 cells analysed in each case, using the FACS440 at the University of Birmingham operated by Miss A Milner.

able to increase H-2K<sup>k</sup> antigen expression on astrocytes, yet was beyond the detection limit of the IFN assay (3 u/ml). That these supernatants did not also increase class II H-2A<sup>k</sup> antigen expression on astrocytes (see table 6.2) may be a reflection of the fact that higher concentrations of IFN- $\gamma$  (in terms of antiviral activity units) are needed to increase class II MHC antigen expression on astrocytes than are needed to increase class I MHC antigen expression. Thus, the data presented in chapter 4 (figures 4.14 and 4.16) show that the lowest concentration of IFN- $\gamma$  which brought about a detectable increase in class II MHC H-2A<sup>k</sup> antigen expression on astrocytes was 1 u/ml, whereas a detectable increase in class I MHC H-2K<sup>k</sup> antigen expression was observed at the lower concentration of 0.01 u/ml. In the present study, supernatants harvested from co-cultures of non-IFN treated BPLSFV-treated astrocytes with SFV-specific T-cells increased H-2K<sup>k</sup> antigen expression on astrocytes to an even higher level, with an increase in mean fluorescence from 27 for untreated astrocytes to 84 for supernatant-treated astrocytes, and an increase in the percentage of cells collected above the 5% background fluorescence marker from 18% for untreated astrocytes to 54% for supernatant-treated astrocytes. The increased ability of this supernatant to increase H-2K<sup>k</sup> antigen expression on astrocytes (as compared to supernatants harvested from co-cultures of non-BPLSFV treated astrocytes with SFV-specific T-cells) is probably due to the presence of IFN- $\gamma$  released by SFV-specific T-cells when stimulated by SFV antigen on the BPLSFV-treated astrocytes. Supernatants harvested from co-cultures of IFN- $\alpha\beta$  treated BPLSFV-treated astrocytes with SFV-specific T-cells, and from co-cultures of IFN- $\gamma$  treated BPLSFV-treated astrocytes with SFV-specific T-cells also increased H-2K<sup>k</sup> antigen expression on astrocytes to a high level, with an increase in mean fluorescence from 27 for untreated astrocytes to 91 for IFN- $\alpha\beta$  treated BPLSFV-treated co-culture supernatant-treated astrocytes and to 77 for IFN- $\gamma$  treated BPLSFV-treated co-culture

supernatant treated astrocytes, and an increase in the percentage of cells collected above the 5% background fluorescence marker from 18% for untreated astrocytes to 58% for IFN- $\alpha\beta$  treated BPLSFV-treated co-culture supernatant treated astrocytes and to 48% for IFN- $\gamma$  treated BPLSFV-treated co-culture supernatants.

The effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  treatment on the ability of BPLSFV-treated G26-24 cells to stimulate SFV-specific T-cell release of IFN- $\gamma$  was also investigated. The data in table 6.4 show the titres of IFN activity detected in supernatants harvested from co-cultures of G26-24 cells with SFV-specific T-cells, and from cultures of G26-24 cells alone. It can be seen that G26-24 cells alone did not release any detectable IFN when treated with BPLSFV. Supernatants harvested from co-cultures of non-BPLSFV treated G26-24 cells (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) with SFV-specific T-cells, and from co-cultures of non-IFN treated BPLSFV-treated and IFN- $\alpha\beta$  treated BPLSFV-treated G26-24 cells with SFV-specific T-cells also contained no detectable IFN activity. In contrast, supernatants harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated G26-24 cells with SFV-specific T-cells contained high levels of IFN activity ( $142 \pm 16$  u/ml). The IFN type appears to be IFN- $\gamma$  since activity was neutralised by the monoclonal antibody to IFN- $\gamma$  (from  $142 \pm 16$  u/ml to  $< 3$  u/ml). The release of IFN- $\gamma$  was also found to be MHC restricted, since WEHI 3b cells (H-2<sup>d</sup>) treated in the same manner did not stimulate the SFV-specific T-cell release of IFN- $\gamma$  (data not shown).

The effect of the supernatants harvested from co-cultures of G26-24 cells with SFV-specific T-cells, and from cultures of G26-24 cells alone, on class II MHC H-2A<sup>b</sup> antigen expression by G26-24 cells was also investigated. H-2A<sup>b</sup> antigen expression was assessed by indirect

Table 6.4

Effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on ability of BPLSFV-treated G26-24 cells to stimulate SFV-specific T-cell release of IFN- $\gamma$

G26-24 cell treatment	Supernatants from co-culture of G26-24 cells with SFV-T-cells		Supernatants from cultures of G26-24 cells alone	
	Alone	+ Anti IFN- $\gamma$	Alone	+ Anti IFN- $\gamma$
IFN- $\gamma$ + BPLSFV	142 $\pm$ 16 <sup>+</sup>	<3	<3	N.D.
BPLSFV alone	<3	N.D. <sup>*</sup>	<3	N.D.
IFN- $\alpha\beta$ + BPLSFV	<3	N.D.	<3	N.D.
IFN- $\gamma$ alone	<3	N.D.	<3	N.D.
IFN- $\alpha\beta$ alone	<3	N.D.	<3	N.D.
Untreated	<3	N.D.	<3	N.D.

<sup>+</sup>: titres refer to mean of duplicate titrations  $\pm$  S.D. units/ml IFN activity

<sup>\*</sup>: N.D. not done

- (i) Experimental protocol as described in methods section M.
- (ii) Cells treated with 1000 u/ml IFN- $\alpha\beta$ , 500 u/ml IFN- $\gamma$  or medium alone.
- (iii) Supernatants were harvested from cultures of G26-24 cells, treated as indicated, in the presence and absence of SFV-specific T-cells (derived from C57 BL/6 mice). IFN activity was assessed and characterized as described in methods section P.



immunofluorescence staining with quantification by flow cytometry. As shown in table 6.5, all supernatants harvested from cultures of G26-24 cells alone had no effect on H-2A<sup>b</sup> antigen expression by G26-24 cells, since the fluorescence of cells treated with these supernatants was essentially identical to background fluorescence. Similarly, all supernatants harvested from co-cultures of G26-24 cells with SFV-specific T-cells (with the exception of supernatant harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated G26-24 cells with SFV-specific T-cells) had no effect on H-2A<sup>b</sup> antigen expression by G26-24 cells. Supernatants harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated G26-24 cells with SFV-specific T-cells markedly increased H-2A<sup>b</sup> antigen expression on G26-24 cells, with an increase in mean fluorescence from 6 for untreated G26-24 cells to 51 for supernatant-treated G26-24 cells and an increase in the percentage of cells collected above the 5% background fluorescence marker from 5% for untreated G26-24 cells to 63% for supernatant-treated G26-24 cells.

The effect of anti-IFN- $\gamma$  monoclonal antibody on the ability of supernatant harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated G26-24 cells with SFV-specific T-cells to increase H-2A<sup>b</sup> antigen expression on G26-24 cells was also investigated. As shown in figure 6.3 (and as discussed above) the supernatant alone markedly increased H-2A<sup>b</sup> antigen expression on G26-24 cells. The anti-IFN- $\gamma$  monoclonal antibody completely inhibited the ability of the co-culture supernatant to increase H-2A<sup>b</sup> antigen expression on G26-24 cells, with a decrease in mean fluorescence from 51 for G26-24 cells treated with supernatant alone to 5 for G26-24 cells treated with supernatant and anti-IFN- $\gamma$  antibody. These observations suggest that IFN- $\gamma$  was the factor present in the co-culture supernatant which increased H-2A<sup>b</sup> antigen expression on the G26-24 cells.

Table 6.5

Effect of supernatants harvested from co-cultures of G26-24 cells with SFV-specific T-cells on class II H-2A<sup>b</sup> antigen expression by G26-24 cells

G26-24 treatment	Fluorescence intensity distribution			
	Supernatant from co-cultures of G26-24 cells with SFV-T-cells		Supernatant from cultures of G26-24 cells alone	
	MN <sup>*</sup>	% <sup>+</sup>	MN	%
IPW- $\gamma$ + BPLSFV	51	63	7	4
BPLSFV alone	7	3	8	5
IPW- $\alpha\beta$ +BPLSFV	7	4	8	3
IPW- $\gamma$ alone	7	2	7	2
IPW- $\alpha\beta$ alone	6	3	7	5
Untreated	6	3	5	4

Background fluorescence (non-supernatant treated, stained for H-2A<sup>b</sup>);

MN = 6, % = 5.

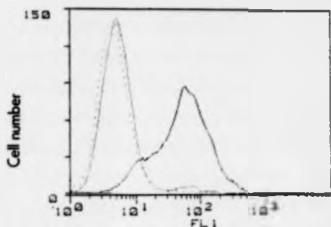
\* MN; Mean fluorescence

+%; Percentage of cells above 5% background fluorescence marker (non-supernatant treated, stained with an irrelevant antibody).

- (1) Supernatants were harvested from wells containing G26-24 cells treated as indicated, and cultured in the presence or absence of SFV-specific T-cells as described in methods section N. Supernatants were then diluted 1:20 prior to addition to G26-24 cells for 48 hours. Class II (H-2A<sup>b</sup>) antigen was then stained by indirect immunofluorescence as described in methods section M.
- (11) 5000 cells analysed in each case.

Figure 6.3

Effect of anti-IFN- $\gamma$  antibody on ability of supernatant harvested from co-cultures of IFN- $\gamma$  treated BFL-SPV-treated G26-24 cells with SPV-T-cells to augment class II (M-2A<sup>b</sup>) antigen expression on G26-24 cells



**Key**

- solid line : co-culture supernatant alone
- spaced dots : co-culture supernatant + anti-IFN- $\gamma$  antibody
- close dots : Untreated

FLI, Fluorescence intensity histogram

- (i) Supernatants were harvested from co-cultures of IFN- $\gamma$  treated BFLSPV-treated G26-24 cells and SPV-T-cells as described in methods section M. Supernatants were then diluted 1:20 prior to addition to G26-24 cells in the presence or absence of a 1:100 dilution of B4-6A2 anti-IFN- $\gamma$  antibody ascites fluid. Class II (M-2A<sup>b</sup>) antigen was stained by indirect immunofluorescence as described in methods section M.
- (ii) 5000 cells were analysed in each case.

The effect of the supernatants on class I MHC H-2D<sup>b</sup> antigen expression by G26-24 cells was also investigated. As shown in table 6.6, all supernatants harvested from cultures of G26-24 cells alone had no effect on H-2D<sup>b</sup> antigen expression, since the fluorescence of cells treated with these supernatants was essentially identical to background fluorescence. Supernatants harvested from co-cultures of non-BPLSFV treated G26-24 cells (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) with SFV-specific T-cells slightly increased H-2D<sup>b</sup> antigen expression on G26-24 cells, with an increase in mean fluorescence from 48 for untreated G26-24 cells to around 122 for supernatant-treated G26-24 cells, and an increase in the percentage of cells collected above the 5% background fluorescence marker from 5% for untreated G26-24 cells to around 16% for supernatant-treated G26-24 cells. The ability of these supernatants to increase H-2D<sup>b</sup> antigen expression on G26-24 cells existed despite the absence of any detectable IFN activity (see table 6.4). The most likely explanation for this data is that the SFV-specific T-cells non-specifically released very small amounts of IFN- $\gamma$  which was able to increase H-2D<sup>b</sup> antigen expression on G26-24 cells, yet was beyond the detection limit of the IFN assay (3 u/ml). That these supernatants did not also increase H-2A<sup>b</sup> antigen expression on G26-24 cells (see table 6.5) may be a reflection of the fact that higher concentrations of IFN- $\gamma$  (in terms of antiviral activity units) are needed to increase class II MHC antigen expression on G26-24 cells than are needed to increase class I MHC antigen expression. Thus, the data presented in chapter 4 (figures 4.19 and 4.23) show that the lowest concentration of IFN- $\gamma$  which brought about a detectable increase in class II MHC H-2A<sup>b</sup> antigen expression on G26-24 cells was 0.5 u/ml, whereas a detectable increase in class I MHC H-2D<sup>b</sup> antigen expression was observed at the lower concentration of 0.05 u/ml. In the present study, supernatants harvested from co-cultures of non-IFN treated BPLSFV-treated G26-24 cells with SFV-

Table 6.6

Effect of supernatants harvested from co-cultures of G26-24 cells with SFV-specific T-cells on class I MHC (H-2D<sup>b</sup>) antigen expression by G26-24 cells

G26-24 treatment	Fluorescence intensity distribution			
	Supernatant from co-cultures of G26-24 cells with SFV-T-cells		Supernatant from cultures G26-24 cells alone	
	MN <sup>*</sup>	% <sup>+</sup>	MN	%
IFN- $\gamma$ + BPLSFV	323	73	51	4
BPLSFV alone	140	24	61	4
IFN- $\alpha\beta$ + BPLSFV	136	23	55	3
IFN- $\gamma$ alone	126	16	59	3
IFN- $\alpha\beta$ alone	109	14	46	2
Untreated	122	16	57	3

Background fluorescence (non-supernatant treated, stained for H-2D<sup>d</sup> antigen): MN = 48, % = 5.

\* MN; Mean fluorescence

+ %; Percentage of cells above 5% background fluorescence marker (as above).

- (1) Figure legend as for table 6.5, except cells were stained for H-2D<sup>b</sup> antigen by indirect immunofluorescence.
- (11) 5000 cells analyzed in each case.

specific T-cells and from co-cultures of IFN- $\alpha\beta$  treated BPLSFV-treated G26-24 cells with SFV-specific T-cells, increased M-2D<sup>b</sup> antigen expression on G26-24 cells to a slightly higher level than for supernatants harvested from co-cultures of non-BPLSFV treated G26-24 cells (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) with SFV-specific T-cells (see table 6.4). The slightly increased ability of these supernatants to increase M-2D<sup>b</sup> antigen expression on G26-24 cells may be due to the presence of a very small amount of IFN- $\gamma$  released by SFV-specific T-cells when stimulated by SFV antigen on the BPLSFV-treated G26-24 cells. Supernatants harvested from co-cultures of IFN- $\gamma$  treated BPLSFV-treated G26-24 cells with SFV-specific T-cells increased M-2D<sup>b</sup> antigen expression to an even higher level, with an increase in mean fluorescence from 48 for untreated G26-24 cells to 323 for supernatant treated G26-24 cells, and an increase in the percentage of cells collected above the 5% background fluorescence marker from 5% for untreated G26-24 cells to 73% for supernatant-treated G26-24 cells. The ability of this supernatant to increase M-2D<sup>b</sup> antigen expression to such an high level is probably due to the presence of the large amount of IFN- $\gamma$  released by the SFV-specific T-cells when stimulated with IFN- $\gamma$  treated BPLSFV-treated G26-24 cells (see table 6.4).

### Discussion

In this chapter the effect of natural IFN- $\alpha\beta$  and recombinant IFN- $\gamma$  treatment on the ability of BPLSFV-treated astrocytes to present SFV antigen to SFV-specific T-cells was investigated. The data obtained show that non-IFN treated BPLSFV-treated astrocytes stimulated SFV-specific T-cells to release IFN- $\gamma$  and that IFN- $\alpha\beta$  pretreatment of astrocytes prior to BPLSFV treatment increased the ability of the cells to stimulate SFV-specific T-cell release of IFN- $\gamma$ . Since at least some non-IFN treated astrocytes display class I MHC antigen on the cell surface (see chapter 4 figure 4.14) and non-IFN treated BPLSFV-treated astrocytes were found to be susceptible to SFV-specific CTL lysis (see chapter 5 figure 5.2) it does at least seem possible that the IFN- $\gamma$  may have been released by class I MHC restricted SFV-specific CTL on recognition of SFV antigen on the astrocytes. That IFN- $\alpha\beta$  pretreatment increased the ability of BPLSFV-treated astrocytes to stimulate SFV-specific T-cell release of IFN- $\gamma$  may be a reflection of the fact that IFN- $\alpha\beta$  treatment increased class I MHC antigen expression on the astrocytes (see chapter 4 figures 4.11 and 4.13) hence the ability to be recognised by SFV-specific CTL. The ability of virus-specific CTL to release IFN- $\gamma$  on recognition of a virus-infected cell has been reported previously in the influenza virus system (Morris *et al.*, 1982; Taylor *et al.*, 1985). The possibility that SFV-specific T-helper cells may have released IFN- $\gamma$  on recognition of SFV antigen on the non-IFN treated BPLSFV-treated and IFN- $\alpha\beta$  treated BPLSFV-treated astrocytes cannot be excluded. However, this does at least seem unlikely since T-helper cells only recognise viral antigen in association with class II MHC antigen (Klein *et al.*, 1981) and neither non-IFN treated nor IFN- $\alpha\beta$  treated astrocytes display any detectable class II MHC antigen on the cell surface (see chapter 4 figure 4.17). The data presented in this chapter also show

IFN- $\gamma$  pretreatment of BPLSFV-treated astrocytes increased the ability of the astrocytes to stimulate SFV-specific T-cell release of IFN- $\gamma$ , and to an even higher level than observed for IFN- $\alpha\beta$  treated BPLSFV-treated astrocytes. That IFN- $\gamma$  pretreatment increased the ability of BPLSFV-treated astrocytes to stimulate SFV-specific T-cell release of IFN- $\gamma$  may again be a reflection of the fact that IFN- $\gamma$  treatment increased class I MHC antigen expression on the astrocytes (see chapter 4 figures 4.12 and 4.14) hence the ability to be recognised by SFV-specific CTL, which release IFN- $\gamma$  (IFN- $\gamma$  treatment was also found to increase the susceptibility of BPLSFV-treated astrocytes to SFV-specific CTL lysis, see chapter 5 figure 5.2). There are several explanations which may account for the observation that the SFV-specific T-cells released higher titres of IFN- $\gamma$  when stimulated with IFN- $\gamma$  treated BPLSFV-treated astrocytes than when stimulated with IFN- $\alpha\beta$  treated BPLSFV-treated astrocytes. The first is that the IFN- $\gamma$  treated astrocytes displayed a higher level of class I MHC antigen on the cell surface than IFN- $\alpha\beta$ -treated astrocytes (see chapter 4 table 4.2) hence were more effectively recognised by the SFV-specific CTL. Alternatively, the induction by IFN- $\gamma$  (but not IFN- $\alpha\beta$ ) of class II MHC antigen expression on astrocytes (see chapter 4 figure 4.17) may have increased the ability of the cells to be recognised by class II MHC restricted SFV-specific T-helper cells, which also released IFN- $\gamma$ . Since class II restricted CTL have now been demonstrated in a number of experimental systems (Sun and Wekarle, 1986; Morrison *et al.*, 1988) it is also possible that the induction by IFN- $\gamma$  (but not IFN- $\alpha\beta$ ) of class II MHC antigen expression on astrocytes may have increased the ability of the cells to be recognised by class II restricted SFV-specific CTL, which released IFN- $\gamma$ . A possible experimental approach which could be used in order to determine which MHC antigens on astrocytes were involved in the stimulation of IFN- $\gamma$  release from SFV-specific T-cells, would be to add



antibodies against class I or class II MHC antigen to the co-cultures of astrocytes with SFV-specific T-cells and assess the effect on the amount of IFN- $\gamma$  released. Similarly, since class I MHC restricted CTL express Lyt-2 antigen on the cell surface and both class II MHC restricted CTL and T-helper cells express L3T4 antigen on the cell surface (Male *et al.*, 1987) antibodies against Lyt-2 and L3T4 could be used in the presence of complement to deplete each of these cell types from the SFV-specific T-cell preparation prior to the addition to the co-culture with astrocytes. A comparison could then be made of the amount of IFN- $\gamma$  released by the Lyt-2 depleted, L3T4 depleted and non-depleted SFV-specific T-cell preparations, when stimulated with astrocytes, in order to determine the phenotype(s) of the SFV-specific T-cells with release IFN- $\gamma$ . An alternative approach would also be to study the release of IFN- $\gamma$  from class I and class II MHC restricted SFV-specific T-cell clones. It is important to note that the astrocyte cultures used for the experiments presented in this chapter also contained (at most) 10% uncharacterised cells. Hence, it is not possible to definitely state that the SFV-specific T-cell release of IFN- $\gamma$  was in response to SFV-antigen on astrocytes, but may have been in response to SFV antigen on the uncharacterised cells also present. It is however interesting to note that Fontana and co-workers demonstrated that cultured rat astrocytes (derived from Lewis rats and which contained less than 10% uncharacterised cells) presented myelin basic protein (MBP) to a MBP-specific T-helper cell line, as assessed by proliferation assay, and observed the antigen-specific and MHC-restricted aggregation of T-cells around cells which were positively identified as astrocytes by anti-GFAP indirect immunofluorescence staining (Fontana *et al.*, 1984; Fiers *et al.*, 1985). The results of these studies also demonstrated that cultured astrocytes did not express any detectable class II MHC antigen on the cell surface (as assessed by indirect immunofluorescence staining) and were only

able to present MBP to the MBP-specific T-helper cells when induced to express class II MHC antigen by either treatment with recombinant IFN- $\gamma$  or by a period of co-culture with the MBP-specific T-cells (which were thought to have released IFN- $\gamma$ ). Thus a correlation existed between the expression of class II MHC antigen on astrocytes and the ability to present MBP to a MBP-specific T-helper cell line. The importance of class II MHC antigen expression on the astrocytes was also demonstrated by the addition of anti-class II MHC antigen monoclonal antibodies which were found to inhibit the ability of astrocytes to present MBP to MBP-specific T-helper cells (Fierz *et al.*, 1985). Subsequent studies by Fontana and co-workers also demonstrated that whilst cultured non-IFN treated murine astrocytes (derived from C57BL/6 mice and which expressed class I MHC H-2K<sup>b</sup> antigen on the cell surface) presented N-iodoacetyl-N-(5-sulfonic-1-naphthyl)-ethylenediamine (AED) hapten to a class I MHC H-2K<sup>b</sup> restricted AED-specific CTL clone, as assessed by proliferation assay, only IFN- $\gamma$  treated astrocytes (hence which expressed class II MHC antigen on the cell surface) presented beef insulin to a class II MHC restricted beef-insulin-specific T-cell hybridoma, as assessed by the release of interleukin-2 (Fontana *et al.*, 1986). Similarly, Takiguchi and Frelinger (1986) demonstrated that cultured murine astrocytes (derived from B10.A mice) were only able to present key-hole limpet haemocyanin (KLH) and egg white lysozyme to appropriate class II MHC-restricted T-cell hybridomas when induced to express class II MHC antigen by treatment with recombinant IFN- $\gamma$ . The importance of class II MHC antigen expression on astrocytes was again demonstrated by the addition of anti-class II MHC antigen monoclonal antibodies which (unlike anti-class I MHC antigen monoclonal antibodies) inhibited the ability of the astrocytes to present KLH to the class II MHC restricted KLH-specific T-cell hybridoma.

since it is currently believed that cytotoxic T-cells and helper T-cells recognise processed foreign antigen fragments which have bound to class I and class II MHC antigen (Bjorkman *et al.*, 1987a, 1987b; Babbitt *et al.*, 1986; Germain, 1986) the observation in the present study that astrocytes treated with a  $\beta$ -propiolactone inactivated preparation of SFV stimulated SFV-specific T-cell release of IFN- $\gamma$  does at least suggest that the astrocytes were able to process SFV antigen into a form which could be recognised by the SFV-specific T-cells. The ability of astrocytes to process antigen has not yet been formally demonstrated although Takiguchi and Frelinger (1986) found that IFN- $\gamma$  treated astrocytes presented KLN antigen to the SKK-43.10 KLN-specific T-cell hybridoma, which only recognises processed KLN antigen.

The data presented in this chapter also show that the IFN- $\gamma$  released by SFV-specific T-cells, when stimulated with BPLSFV-treated astrocytes, increased both class I and class II MHC antigen expression on astrocytes. These observations suggest positive feedback mechanism whereby the immune response may escalate in a virus-infected brain. Thus, on recognition of viral antigen on an astrocyte (which is expressing class I or class II MHC antigen on the cell surface) virus-specific CTL and T-helper cells release IFN- $\gamma$  which then further increases class I and class II MHC antigen expression on astrocytes and hence enhances the presentation of viral antigen to T-cells. IFN- $\gamma$  released by virus-specific T-cells on recognition of viral antigen would also inhibit viral replication and thereby contain the spread of virus within the brain.

In a series of experiments it was found that neither BPLSFV-treated astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) nor BPLSFV-treated syngeneic C3H/He mouse splenocytes (used as an experimental

positive control) stimulated any detectable SFV-specific T-cell proliferation, as assessed by the incorporation of [<sup>3</sup>H]thymidine. In contrast, BPLSFV-treated astrocytes (either non-IFN treated, IFN- $\alpha\beta$  treated or IFN- $\gamma$  treated) were regularly found to stimulate SFV-specific T-cell release of IFN- $\gamma$ , indicating that the SFV-specific T-cells were able to recognise SFV antigen on the astrocytes. That no SFV-specific T-cell proliferation could be detected does not appear to have been due to a deficiency in T-cell interleukin-2 production (IL-2, T-cell growth factor) since the addition of recombinant IL-2 had no effect. Astrocytes have also been previously shown to release interleukin-1 (IL-1, T-cell activation factor: Fontana *et al.*, 1982). That BPLSFV-treated syngeneic C3H/He mouse splenocytes also did not stimulate SFV-specific T-cell proliferation, suggests that the SFV-specific T-cell effector population used was unable to undergo proliferation. A possible explanation may be that the frequency of responder SFV-specific T-cells in the effector population used was too low to enable the detection of proliferation in the assay system used. A possible future experimental approach would be to produce an SFV-specific T-cell line which is highly enriched with SFV-specific T-cells. This could be achieved by the successive restimulation of the SFV-specific T-cell effector population with SFV antigen *in vitro* (for example, according to the method of Ben-Nun *et al.* (1981)).

The data presented in this chapter also show that the astrocyte cultures released IFN- $\alpha\beta$  when treated with BPLSFV or infected with SFV. The ability of astrocytes to release IFN- $\alpha\beta$  has also been reported previously by Tadaschi and co-workers who treated astrocytes with the interferon inducer polyribonucleosinic-polyribocytidylic acid (Tadaschi *et al.*, 1986). In the present study, IFN- $\alpha\beta$  released from BPLSFV-treated astrocytes was also found to slightly increase class I MHC antigen expression on astrocytes.

This observation suggests that IFN- $\alpha\beta$  released from SFV-infected astrocytes within an SFV-infected brain may increase class I MHC antigen expression on astrocytes, and hence enhance the presentation of SFV antigen to SFV-specific CTL. The ability of inactivated virus to induce IFN- $\alpha\beta$  production has also been reported previously in other systems (Meager and Burke, 1972).

The effect of IFN- $\alpha\beta$  and IFN- $\gamma$  treatment on the ability of BPLSFV-treated G26-24 oligodendrogloma cells to present SFV antigen to SFV-specific T-cells was also investigated. The data obtained show that neither non-IFN treated BPLSFV-treated G26-24 cells nor IFN- $\alpha\beta$  treated BPLSFV-treated G26-24 cells stimulated SFV-specific T-cells to release IFN- $\gamma$ . Since at least some non-IFN treated G26-24 cells display class I MHC antigen on the cell surface (see chapter 4 figures 4.18 and 4.19) these observations suggest that in this system the class I MHC restricted SFV-specific CTL did not release IFN- $\gamma$ . In contrast, IFN- $\gamma$  treated BPLSFV-treated G26-24 cells clearly stimulated SFV-specific T-cells to release IFN- $\gamma$ . Since only IFN- $\gamma$  treated G26-24 cells display class II MHC antigen on the cell surface (see chapter 4 figure 4.24) these data very obviously suggest that in this system the release of IFN- $\gamma$  from SFV-specific T-cells was class II MHC restricted. Thus, either class II MHC restricted SFV-specific T-helper cells or class II MHC restricted SFV-specific CTL may have released IFN- $\gamma$  on recognition of SFV antigen on G26-24 cells. The data presented in this chapter also show that the IFN- $\gamma$  released by SFV-specific T-cells, when stimulated with IFN- $\gamma$  treated BPLSFV-treated astrocytes, increased both class I and class II MHC antigen expression on G26-24 cells. The observation that IFN- $\gamma$  treated BPLSFV-treated G26-24 oligodendrogloma cells appear to participate in class II MHC restricted T-cell responses does not reflect the situation with normal murine oligodendrocytes. Thus,

IFN- $\gamma$  treatment does not induce class II MHC antigen expression on normal murine oligodendrocytes either in vitro (Suzumura et al., 1986) or in vivo (Wong et al., 1984) and IFN- $\gamma$  treated oligodendrocytes (derived from newborn B10.A mice) do not present either KLN or egg white lysozyme to appropriate class II MHC restricted T-cell hybridomas (Takiguchi and Frelinger, 1986).

In summary, IFN treatment increased the ability of BPLSFV-treated astrocytes and BPLSFV-treated G26-24 cells to present SFV antigen to SFV-specific T-cells, as assessed by the release of IFN- $\gamma$ . The increase in ability to present SFV antigen to SFV-specific T-cells was also found to correlate with an increase in the level of class I or class II MHC antigen expression on the astrocytes and G26-24 cells. It is however important to note that the increased ability of BPLSFV-treated astrocytes and BPLSFV-treated G26-24 cells to present SFV antigen to SFV-specific T-cells may not have been due solely to the IFN-induced increase in MHC antigen expression, since other membrane molecules may have played a role in the interaction between the antigen presenting cells and SFV-specific T-cells (for example, LFA-1, ICAM-1, LFA-3, CD2 - for discussion see chapter 5) whose roles were not investigated in the present studies.

## Chapter 7

Presence of IFN within the brains of SFV-infected C3H/He mice, effect on brain cell MHC antigen expression and preliminary studies using R4-6A2 monoclonal antibody to determine the role of endogenously produced IFN- $\gamma$

### Introduction

In the previous chapters, data were presented which show that IFN- $\alpha\beta$  and IFN- $\gamma$  modulate both MHC antigen expression on brain cells and the ability of SFV-infected brain cells to participate in T-cell mediated immune reactions. It is however important to note that all of these studies were performed in vitro, hence may not have any relevance to the situation in vivo during SFV-infection of mice. In this chapter the studies are extended to the in vivo system. As mentioned in chapter 1, a number of studies have shown that infection of mice with SFV results in the appearance of IFN- $\alpha\beta$  in the serum and brain (Bradish and Allner, 1972; Bradish et al., 1975a; Oaten et al., 1976; Fleming, 1977). That IFN- $\alpha\beta$  has a protective role was shown by experiments in which intravenous administration of anti-IFN- $\alpha\beta$  serum immediately following infection with low doses of SFV was found to result in an earlier and increased mortality (Fauconnier, 1970, 1971). To date, no studies have monitored levels of IFN- $\gamma$  present in SFV-infected mice, or have used anti-IFN- $\gamma$  antibody to determine the role of endogenously produced IFN- $\gamma$ . In this chapter the effect of avirulent A7(74) SFV infection of C3H/He mice on both the amount of IFN- $\alpha\beta$  and IFN- $\gamma$  present within the brain and the level of MHC antigen expressed by cells within the brain is investigated. Furthermore, to determine the role of endogenously produced IFN- $\gamma$ , R4-6A2 rat-anti-murine IFN- $\gamma$  monoclonal antibody (Spitalny and Havell, 1984) is administered to

C3H/He mice, prior to infection with the avirulent A7(74) strain of SFV, and the effect on the clinical course of SFV disease monitored.



## Results

### Presence of IFN within the brains of SFV-infected C3H/He mice and effect on brain cell MHC antigen expression

Adult C3H/He mice were intranasally infected with the avirulent A7(74) strain of SFV, and at various times post-infection, brain extracts prepared from duplicate mice. Brain extracts were then treated with  $\beta$ -propiolactone (BPL) to inactivate live virus prior to IFN assay in the presence and absence of monoclonal antibody to IFN- $\gamma$ . BPL treatment does not affect the activity of IFN- $\alpha\beta$  or IFN- $\gamma$  (Barrett *et al.*, 1984). The data in table 7.1 show the titres of IFN present in SFV-infected C3H/He mouse brain. It can be seen that IFN was present in large amounts at day 3 post-infection and reached a maximal level around day 5 post-infection after which time the amount present declined. Brains harvested from mice at day 10 post-infection (or later) did not contain any detectable IFN. Titration of brain extracts in the presence of anti-IFN- $\gamma$  monoclonal antibody had no significant effect on the amount of IFN detected ( $p < 0.05$ , see methods section F). The activity of an IFN- $\gamma$  preparation was neutralised by the anti-IFN- $\gamma$  monoclonal antibody in a control experiment. In a separate experiment, brain extracts dialysed against pH2 glycine-HCl buffer were found to contain high levels of IFN activity (data not shown). These data suggest that IFN- $\alpha\beta$  was present in the brain extracts. Since a  $0.5 \log_{10}$  units difference in IFN titre was required for statistical significance (see methods section F) it follows that one could have expected the anti-IFN- $\gamma$  antibody to significantly reduce the titre of IFN detected only if IFN- $\gamma$  was present in at least  $0.5 \log_{10}$  units excess over IFN- $\alpha\beta$ . Hence, the possibility cannot be excluded that at least some IFN- $\gamma$  may have been present.

Table 7.1

Time course of titres of IFN present in JEV-infected adult C3H/He mouse brain

Day post-infection brain harvest		Total IFN activity	IFN activity in presence of anti-IFN- $\gamma$
0	A <sup>+</sup>	<1.60*	<1.60
	B	<1.60	<1.60
3	A	2.25	2.65
	B	2.95	3.00
5	A	2.95	2.85
	B	3.40	3.30
7	A	1.60	1.60
	B	2.00	2.25
10	A	<1.60	<1.60
	B	<1.60	<1.60
14	A	<1.60	<1.60
	B	<1.60	<1.60
IFN- $\alpha\beta$ sample		3.60	3.80
IFN- $\gamma$ sample		2.40	<1.60

\*. A and B refer to brains from duplicate mice harvested on day indicated.

\*. refer to  $\log_{10}$  IFN units for each brain.

- (i) 5 to 6 week-old C3H/He mice were intranasally infected with  $6.4 \times 10^5$  pfu (ILD<sub>50</sub>) A7(74) SFV as described in methods section C. At various times post-infection brains were harvested from duplicate mice and an extract prepared as described in methods section E. Brain extracts (and control samples of IFN- $\alpha\beta$  and IFN- $\gamma$ ) were then treated with  $\beta$ -propiolactone to inactivate live virus (Barrett *et al.*, 1984) prior to IFN assay in the presence and absence of B4-6A2 monoclonal antibody to IFN- $\gamma$ .
- (ii) Differences in titre greater than  $0.5 \log_{10}$  units are statistically significant (see methods section F).

To determine whether SFV-infection of adult C3H/He mice had any effect on the level of MHC antigen expressed by cells within the brain, sections were prepared from brains harvested at day 0,3,5,7,10 and 14 post-infection and class I (H-2K<sup>k</sup>) and class II (H-2A<sup>k</sup>) MHC antigens stained by the indirect immunoperoxidase technique. Indirect immunoperoxidase staining was performed using a kit (K660) obtained from Dako Ltd (High Wycombe, UK) and sections were cut from snap-frozen tissues as described in the instruction sheet supplied. However, in a series of experiments, no specific staining of class I or class II MHC antigen was observed, since sections stained with anti-H-2K<sup>k</sup> or anti-H-2A<sup>k</sup> antibody always showed a similar pattern of staining as sections stained with an irrelevant antibody (either anti-H-2D<sup>d</sup> or anti-H-2K<sup>d</sup>) (sections not shown). The non-specific staining was not due to the presence of Fc receptors since these were blocked with a normal swine serum. Similarly, non-specific staining was not due to the presence of endogenous peroxidase activity since this was suppressed by submerging the sections in methanolic hydrogen peroxide. Brains harvested from SFV-infected adult C3H/He mice were also mechanically disaggregated and subjected to trypsinisation (see methods section F) to produce a single-cell suspension of brain cells, since MHC antigens may then be stained by indirect immunofluorescence and quantified by flow cytometry. However, it was not found possible to prepare a single-cell suspension from adult C3H/He brains using this protocol.

To determine whether IFN present within the brains of SFV-infected adult C3H/He mice had the ability to increase MHC antigen expression on brain cells, extracts prepared from the brains of SFV-infected mice were added to cultures of astrocytes and the effect on class I (H-2K<sup>k</sup>) and class II (H-2A<sup>k</sup>) MHC antigen expression investigated, via indirect immunofluorescence

Table 7.2

Effect of brain extracts from A7(74) SFV-infected adult C3H/He mice on class I MHC M-2K<sup>k</sup> antigen expression by cultured astrocytes

Fluorescence intensity distribution data

Day post-infection brain harvest	Treated with brain 'A'	Treated with brain 'B'
0	7* (4%) <sup>*</sup>	7 (5%)
3	28 (46%)	35 (61%)
5	61 (78%)	69 (85%)
7	28 (47%)	81 (87%)
10	29 (47%)	19 (30%)
14	9 (12%)	12 (19%)
untreated	7 (5%)	
treated with IFN- $\alpha\beta$	182 (94%)	
treated with IFN- $\gamma$	425 (94%)	

\*; data outside brackets refer to mean fluorescence

\*; data inside brackets refer to percentage of cells collected above a 5% background fluorescence marker for untreated astrocytes stained with anti-M-2K<sup>k</sup>.

- (i) Adult C3H/He mice were infected with A7(74) and a brain extract prepared (from duplicate mice, brain 'A' and 'B') as described for Table 7.1. Brain extracts were added to astrocyte cultures at a 1:6 dilution. 1000 u/ml IFN- $\alpha\beta$  and 100 u/ml IFN- $\gamma$  were added to control cultures (both IFN- $\alpha\beta$  and IFN- $\gamma$  were also treated with  $\beta$ -propiolactone). After 48 hours H-2K<sup>k</sup> antigen on astrocytes was stained by indirect immunofluorescence and quantitated by flow cytometry.
- (ii) 10 000 cells were analysed in each case.

staining with quantification by flow cytometry. The data in table 7.2 show the effect of brain extracts on class I MHC H-2K<sup>k</sup> antigen expression by cultured astrocytes. It can be seen that brain extracts prepared from mice at day 0 post-infection (i.e. immediately after infection) had no effect on H-2K<sup>k</sup> antigen expression by astrocytes, since the fluorescence of untreated cells and cells treated with day 0 brain extracts were identical. In contrast, brain extracts prepared from mice at day 3, 5 and 7 post-infection markedly increased H-2K<sup>k</sup> antigen expression on astrocytes, and to a level which correlated with the amount of IFN present in the brain extracts (see table 7.1). Similarly, brain extracts prepared from mice at day 10 and 14 post-infection also increased H-2K<sup>k</sup> antigen expression on astrocytes. The ability of day 10 and 14 post-infection brain extracts to increase H-2K<sup>k</sup> antigen expression on astrocytes existed despite the absence of detectable IFN (see table 7.1). The increased fluorescence of brain extract treated astrocytes was specific for staining H-2K<sup>k</sup> antigen since the fluorescence of brain extract treated astrocytes stained with an irrelevant antibody was identical to that of untreated astrocytes stained with the same antibody (data not shown). The effect of the brain extracts on class II MHC H-2A<sup>k</sup> antigen expression on astrocytes was also investigated. The data obtained clearly showed that brain extracts prepared at day 0, 3, 5, 7, 10 and 14 post-infection had no effect on H-2A<sup>k</sup> antigen expression by astrocytes, since the fluorescence of untreated cells and cells treated with brain extract were identical (data not shown).

Newborn (0 to 15 hours old) C3H/He mice were intraperitoneally infected with the avirulent A7(74) strain of SFV and the production of IFN within the brain investigated. The reason for using newborn mice was that a single-cell suspension can be prepared from the brains of newborn mice (unlike adult mice) hence it was possible to study MHC antigen expression

Table 7.3

Time-course of titres of IFN- $\alpha\beta$  and SFV present in SFV-infected newborn C3H/He mouse brain

Time Post-infection	SFV*	IFN- $\alpha\beta$ †
0 hr	$<10^2$	$<3$
24 hr	$2.8 \times 10^7$	$31 \pm 10$
42 hr	$4.1 \times 10^8$	$880 \pm 220$

\* : refers to mean p.f.u./brain

† : refers to mean  $\pm$  S.D. IFN- $\alpha\beta$  activity per brain

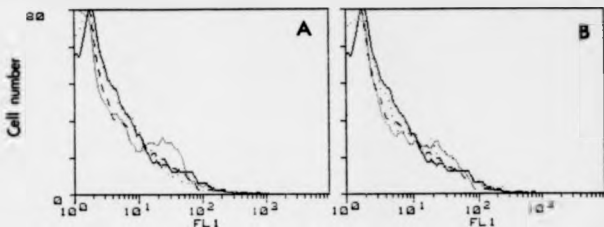
- (1) Newborn C3H/He mice were infected intraperitoneally with  $8 \times 10^3$  p.f.u. A7(74) SFV in 50  $\mu$ l GNEM medium. At 0, 24 and 42 hours post-infection brains were harvested from 5 replicate mice as described in methods section E. SFV present in the subsequent brain suspension was then assayed by plaque assay. Brain suspension was dialysed twice against pH 2 glycine-HCl overnight and once against GNEM medium prior to IFN assay.



on cells within an SFV-infected brain via indirect immunofluorescence staining with quantitation by flow cytometry. The data in table 7.3 show the titres of IFN- $\alpha\beta$  and SFV present in the brains of SFV-infected newborn C3H/He mice. It can be seen that the amount of IFN- $\alpha\beta$  within the brains increased throughout the course of infection and correlated with the titre of virus present. No IFN- $\alpha\beta$  or SFV were detected in the brains of newborn mice treated intraperitoneally with medium alone (data not shown). In a separate experiment all SFV-infected newborn mice died at around 48 hours post-infection (data not shown). Figures 7.1a and b show the effect of SFV-infection on class I (H-2K<sup>k</sup>) and class II (H-2A<sup>k</sup>) MHC antigen expression by cells within the brains of newborn C3H/He mice. As shown in figure 7.1a, cells harvested from the brains of uninfected mice displayed no detectable H-2K<sup>k</sup> antigen since the fluorescence of anti-H-2K<sup>k</sup> stained cells was identical to that of the same cells stained with an irrelevant antibody (anti-H-2D<sup>d</sup>). In contrast, at least some cells harvested from the brains of SFV-infected mice displayed H-2K<sup>k</sup> antigen since the fluorescence of a small proportion of anti-H-2K<sup>k</sup> stained cells was clearly higher than for the same cells stained with an irrelevant antibody. Statistical analysis of the fluorescence data showed that 6% of cells harvested from the brains of SFV-infected mice displayed H-2K<sup>k</sup> antigen on the cell-surface. As shown in figure 7.1b, cells harvested from the brains of uninfected mice also displayed no detectable H-2A<sup>k</sup> antigen since the fluorescence of anti-H-2A<sup>k</sup> stained cells was identical to that of the same cells stained with an irrelevant antibody. A very small proportion of cells harvested from the brains of SFV-infected mice appeared to display low levels of H-2A<sup>k</sup> antigen since the fluorescence of some anti-H-2A<sup>k</sup> stained cells was slightly higher than for the same cells stained with an irrelevant antibody. Statistical analysis of the fluorescence data showed that 2% of cells harvested from the brains of SFV-infected mice displayed

Figure 7.1

Effect of A7(74) SPV infection of newborn mice on class I and class II  
MHC antigen expression by cells within the brain



FL1: Fluorescence intensity channel

- (A) solid line : uninfected, stained with an irrelevant antibody  
spaced dots : uninfected, stained with anti-H-2K<sup>b</sup>  
close dots : infected, stained with anti-H-2K<sup>b</sup>  
dashed line : infected, stained with an irrelevant antibody
- (B) solid line : uninfected, stained with an irrelevant antibody  
spaced dots : uninfected, stained with anti-H-2A<sup>k</sup>  
close dots : infected, stained with anti-H-2A<sup>k</sup>  
dashed line : infected, stained with an irrelevant antibody

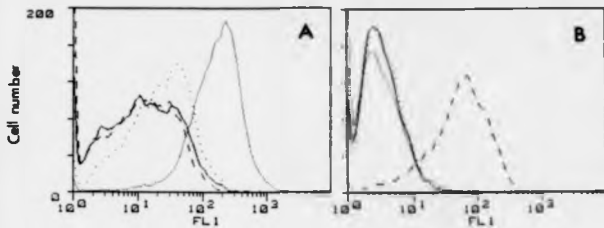
- (i) Newborn C3H/He mice were infected intraperitoneally with A7(74) SFV or treated with medium alone, as described in methods section C. At 42 hours post-infection brains were removed and a single cell-suspension prepared as described in methods section F. Brain cells were then stained with anti-H-2K<sup>k</sup>, anti-H-2A<sup>k</sup> or anti-H-2D<sup>d</sup> (irrelevant antibody) by indirect immunofluorescence and analysed by flow cytometry.
- (ii) Debris was excluded from analysis using a forward scatter threshold as shown by Wong *et al.* (1984). 5 000 cells were analysed in each case.

H-2A<sup>k</sup> antigen on the cell-surface. In a separate experiment, the cell-size and granularity profile (determined by constructing data analysis dot-plots of forward scatter against side scatter) for cells harvested from the brains of newborn mice was found to be similar to the profile for blood cells alone (data not shown). Hence, it is not possible from the data obtained to determine whether the cells which expressed H-2K<sup>k</sup> and H-2A<sup>k</sup> antigen were indeed brain cells (neurons, astrocytes, etc) or cells derived from blood.

Brain extracts were also prepared from SFV-infected newborn C3H/He mice, added to cultures of astrocytes, and the effect on class I (H-2K<sup>k</sup>) and class II (H-2A<sup>k</sup>) MHC antigen expression investigated via indirect immunofluorescence staining with quantification by flow cytometry. As shown in figure 7.2a, brain extract prepared from uninfected mice very slightly increased H-2K<sup>k</sup> antigen expression on a small proportion of astrocytes, since the fluorescence of astrocytes treated with brain extract was slightly higher than for untreated astrocytes. Brain extracts prepared from mice at 24 hours post-infection clearly increased H-2K<sup>k</sup> antigen expression on a greater proportion of astrocytes, and brain extract prepared at 42 hours post-infection strongly increased H-2K<sup>k</sup> antigen expression on essentially all astrocytes present. Statistical analysis of the fluorescence data showed that brain extracts prepared from uninfected mice increased H-2K<sup>k</sup> antigen expression on 3% of astrocytes, and that brain extracts prepared from mice at 24 and 42 hours post-infection increased H-2K<sup>k</sup> antigen expression on 8% and 78% of astrocytes, respectively. The increased fluorescence of brain extract treated astrocytes was specific for staining H-2K<sup>k</sup> antigen since the fluorescence of brain extract treated astrocytes stained with an irrelevant antibody (anti-H-2D<sup>d</sup>) was identical to that of untreated astrocytes stained with the same antibody (data not

Figure 7.2

Effect of brain extracts from A7(74) SFV-infected newborn mice on class I and class II MHC antigen expression by cultured astrocytes



FLI: Fluorescence intensity channel

(A) stained with anti-H-2k

dashed line : untreated

solid line : treated with brain extract from uninfected mice

spaced dots : treated with brain extract from 24 hour-infected mice

close dots : treated with brain extract from 42 hour-infected mice

(B) stained with anti-I-Ek

close dots : untreated

solid line : treated with brain extract from 24 hour-infected mice

spaced dots : treated with brain extract from 42 hour-infected mice

dashed line : treated with 100 u/ml IFN- $\gamma$  (experimental positive control)

- (i) Newborn C3H/He mice were infected intraperitoneally with A7(76) SFV as described in methods section C. At 24 and 42 hours post-infection brains were harvested and an extract prepared as described in methods section E. Brain extracts (and a control sample of IFN- $\gamma$ ) were then treated with  $\beta$ -propiolactone to inactivate live virus (Barrett *et al.*, 1984) prior to addition to astrocyte cultures at a 1:6 dilution. H-2K<sup>k</sup> and H-2A<sup>k</sup> antigens were stained by indirect immunofluorescence after 48 hours.
- (ii) 10 000 cells analysed in each case.

shown). As shown in figure 7.2b, brain extracts prepared from uninfected mice, and from mice at 24 and 42 hours post-infection, had no effect on H-2A<sup>k</sup> antigen expression by astrocytes since the fluorescence of brain extract treated astrocytes and untreated astrocytes were identical.

The effect of brain extracts from SFV-infected newborn and adult C3H/He mice on the susceptibility of astrocytes to lysis by alloreactive CTL was also investigated. As shown in figure 7.3, treatment with brain extracts prepared from both SFV-infected newborn and adult mice increased the susceptibility of astrocytes to lysis by alloreactive CTL, indicating that the brain extracts increased the ability of astrocytes to participate in class I MHC restricted T-cell mediated immune reactions. This increased ability may be due to an increased level of functional class I MHC antigen and/or adhesion molecule display on the cells. The killing by alloreactive CTL was H-2<sup>k</sup> specific since L929 cells (H-2<sup>k</sup>) were lysed whereas WEHI 3b cells (H-2<sup>d</sup>) and G26-24 cells (H-2<sup>b</sup>) were not.

Preliminary studies using the R4-6A2 rat-anti-murine IFN- $\gamma$  monoclonal antibody to determine the role of IFN- $\gamma$  produced endogenously during SFV-infection of C3H/He mice

C3H/He mice (5 to 6 week-old) were treated intraperitoneally with R4-6A2 rat-anti-murine IFN- $\gamma$  monoclonal antibody (on day 0 and 5) and at various times post-treatment serum harvested from duplicate mice. The titre of antibody present in serum was then assessed by IFN- $\gamma$  neutralisation assay as described in methods section P. As shown in table 7.4, serum harvested from mice between day 1 and 14 post-treatment contained high titres of IFN- $\gamma$  neutralising activity, indicating that high titres of R4-6A2 antibody were present. Differences in neutralisation titre of  $10^{0.5}$  (or greater)

Table 7.4

Persistence of R4-6A2 anti-IFN- $\gamma$  monoclonal antibody in the serum of adult C3H/He mice

Day of serum harvest*	Neutralisation titre of harvested serum <sup>†</sup>	
	mouse 'A'	mouse 'B'
Prior to treatment	$<10^2$	$<10^2$
day 1	$10^4$	$>10^4$
2	$>10^4$	$>10^4$
4	$10^{3.8}$	$>10^4$
6	$>10^4$	$>10^4$
8	$10^4$	$10^4$
10	$>10^4$	$>10^4$
14	$10^{3.4}$	$10^{3.5}$

<sup>†</sup>: Neutralisation titre of serum refers to the dilution of serum which neutralised 50% of the antiviral activity of a IFN- $\gamma$  standard preparation (titre 1000 u/ml).

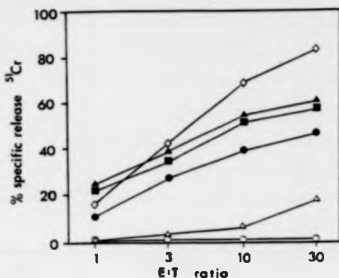
\*: Refers to day after first treatment with R4-6A2 antibody.



- (i) 200  $\mu$ l of B4-6A2 anti-IFN- $\gamma$  monoclonal antibody ascites fluid (neutralising titre  $10^{3.5}$ ) was administered intraperitoneally to 5 to 6 week-old C3H/He mice on day 0 as described in methods section D. Serum was then harvested from duplicate mice at various times post-treatment, as described in methods section E. A further 200  $\mu$ l of B4-6A2 ascites fluid was also administered intraperitoneally on day 5 post-treatment.
- (ii) The neutralisation titre of serum was assessed as described in methods section F.

Figure 7.3

Effect of brain extracts from SFV-infected newborn and adult C3H/He mice on susceptibility of cultured astrocytes to alloreactive CTL lysis



Key

- (●—●) Astrocytes (H-2<sup>k</sup>) untreated
- (▲—▲) Astrocytes (H-2<sup>k</sup>) + extract from newborn mouse brain
- (■—■) Astrocytes (H-2<sup>k</sup>) + extract from adult mouse brain
- (◇—◇) L929 cells (H-2<sup>k</sup>) untreated
- (△—△) G26-24 cells (H-2<sup>b</sup>) untreated
- (○—○) WEHI-3b cells (H-2<sup>d</sup>) untreated

- (i) Brain extracts were prepared at 42 hours post-infection from newborn C3H/He mice, and on day 5 post-infection from adult mice, as described in methods section E. Brain extracts were then dialysed twice against pH2 buffer and once against DMEM medium, prior to addition to astrocyte cultures at a 1:6 dilution, for 48 hours prior to alloreactive CTL assay.
- (ii) Susceptibility to alloreactive CTL lysis was measured in a 44 hour chromium release assay using Balb/c-anti-C3H/He (H-2<sup>d</sup>-anti-H-2<sup>k</sup>) CTL, as described in methods section M.

are statistically significant ( $p < 0.05$ , see methods section F). However, since the neutralisation titres were often found to be beyond the upper detection limit of the assay used ( $10^4$ ) it is not possible to statistically compare many of the neutralisation titres for serum harvested. It can however be seen that the neutralisation titre of serum did not drop significantly ( $p < 0.05$ ) below  $10^4$  until day 14 post-treatment, at which time neutralisation titres of  $10^{3.6}$  and  $10^{3.5}$  were observed. Serum harvested from mice prior to treatment with R4-6A2 antibody did not contain any detectable IFN- $\gamma$  neutralising activity. In a preliminary experiment, brain extracts prepared from mice treated with R4-6A2 antibody were found to contain very low titres of IFN- $\gamma$  neutralising activity (data not shown).

The effect of R4-6A2 rat-anti-murine IFN- $\gamma$  monoclonal antibody on the clinical course of SFV disease in mice was also investigated. In particular, C3H/He mice (5 to 6 week-old) were intraperitoneally treated with R4-6A2 antibody (on day 0 and 5) and intranasally infected with ILD<sub>50</sub> of the avirulent A7(74) strain of SFV (on day 0). Control mice were not treated with R4-6A2 antibody and were infected with SFV alone. The time course of development of clinical symptoms of SFV disease and time of death were then studied. As shown in table 7.5, intraperitoneal treatment with R4-6A2 antibody had no effect on the percentage of mice surviving infection with SFV. Thus, two non-R4-6A2 antibody treated SFV-infected mice and three R4-6A2 antibody treated SFV-infected mice survived infection. No difference in the time of development of clinical symptoms of SFV disease (i.e. hunched posture, rear leg paralysis and full paralysis) or time of death was observed for non-R4-6A2 antibody treated SFV-infected mice and R4-6A2 antibody treated SFV-infected mice (data not shown). Uninfected control mice (both untreated and treated with R4-6A2 antibody) remained healthy throughout the experiment. In a preliminary experiment, no

Table 7.5

Effect of anti-IFN- $\gamma$  antibody on number of C3H/He mice surviving infection with A7(74) SFV

Treatment antibody/virus	Number of mice surviving infection
untreated/uninfected	8/8
untreated/infected with SFV	2/8
R4-6A2/infected with SFV	3/8
R4-6A2/uninfected	8/8

- (1) 200  $\mu$ l of R4-6A2 anti-IFN- $\gamma$  monoclonal antibody ascites fluid (neutralising titre  $10^3$ , see methods section I) was administered intraperitoneally to 6-week-old C3H/He mice as described in methods section D. Mice were then infected with  $6.4 \times 10^5$  pfu (ILD<sub>50</sub>) A7(74) SFV, as described in methods section C. On day 5 post-infection a further 200  $\mu$ l of R4-6A2 ascites fluid was administered intraperitoneally. Clinical symptoms were scored each day until day 13 post-infection.

difference was observed in the titres of IFN- $\alpha\beta$  detected in brain extracts prepared from non-R4-6A2 antibody treated SFV-infected mice and R4-6A2 antibody treated SFV-infected mice (data not shown).

To determine whether R4-6A2 monoclonal antibody had the ability to neutralise the activity of IFN- $\gamma$  *in vivo* it was first necessary to set up a model system in which an effect of IFN- $\gamma$  *in vivo* could be monitored. The ability of R4-6A2 antibody to block this effect of IFN- $\gamma$  *in vivo* could then be investigated in subsequent experiments. Previous studies by Talmadge *et al.* (1985) had demonstrated that intravenous administration of recombinant murine IFN- $\gamma$  (a non-glycosylated bacterial product was used) increased the level of natural killer (NK) cell activity in the spleens of C3H/HeN mice. The ability of IFN- $\gamma$  to increase NK cell activity *in vivo* thereby provided a simple model system. However, since Talmadge and co-workers used a non-glycosylated form of IFN- $\gamma$  it was necessary to establish whether the 'natural' glycosylated form of recombinant IFN- $\gamma$  also increased NK cell activity *in vivo* (since the aim of these studies is to determine whether R4-6A2 antibody neutralises the effect of the 'natural' form of IFN- $\gamma$  *in vivo*). C3H/He mice were thereby intraperitoneally treated with various doses of glycosylated recombinant IFN- $\gamma$  in PBS (IFN- $\gamma$  produced by Chinese hamster ovary cells, see materials section E), or PBS alone, and the NK cell activity of splenic lymphocytes examined in a chromium release assay with Yac-1 cells as targets, as described in methods section M. As shown in table 7.6, splenic lymphocytes harvested from C3H/He mice treated with PBS alone showed moderate levels of NK cell activity at all effector to target ratios studied. Treatment of C3H/He mice with  $2.5 \times 10^2$  u IFN- $\gamma$  was found to very slightly reduce the NK cell activity of splenic lymphocytes. In contrast, treatment with  $2.5 \times 10^3$  u IFN- $\gamma$  somewhat increased the NK cell activity of splenic lymphocytes and  $2.5 \times 10^4$  u IFN- $\gamma$  very markedly

Table 7.6

Effect of IFN- $\gamma$  on the natural killer cell activity of C3H/He mouse splenic lymphocytes

Treatment	Effector : Target ratio		
	60:1	30:1	15:1
PBS	29 $\pm$ 6	23 $\pm$ 4	16 $\pm$ 3
2.5 $\times$ 10 <sup>2</sup> U IFN- $\gamma$	22 $\pm$ 3	13 $\pm$ 1	8 $\pm$ 2
2.5 $\times$ 10 <sup>3</sup> U IFN- $\gamma$	39 $\pm$ 5	31 $\pm$ 2	22 $\pm$ 2
2.5 $\times$ 10 <sup>4</sup> U IFN- $\gamma$	57 $\pm$ 3	42 $\pm$ 6	44 $\pm$ 4

- (i) The appropriate concentration of IFN- $\gamma$  in 200  $\mu$ l PBS, or 200  $\mu$ l PBS alone, was administered intraperitoneally to duplicate 9 week-old C3H/He mice, as described in methods section D. After 19 hours the spleens were removed and the lymphocytes harvested over lymphoprep as described in methods section L. The non-specific cytotoxic activity of the lymphocytes was then tested in a 4.5 hr chromium release assay with Yac-1 as target cells, as described in methods section M.
- (ii) Data are presented as mean specific percentage lysis  $\pm$  standard deviation for duplicate mice.

increased the NK cell activity of splenic lymphocytes. The effect of RA-6A2 antibody on the ability of IFN- $\gamma$  to increase the NK cell activity of C3H/He mouse splenic lymphocytes was then investigated. In particular, C3H/He mice were treated intraperitoneally with either RA-6A2 antibody or PBS alone, and 24 hours later treated intraperitoneally with IFN- $\gamma$ . The NK cell activity of splenic lymphocytes was then assessed as before. As shown in table 7.7, intraperitoneal treatment with RA-6A2 antibody had no effect on the ability of IFN- $\gamma$  to increase the NK cell activity of splenic lymphocytes, since the NK cell activity of splenic lymphocytes from RA-6A2 antibody treated IFN- $\gamma$  treated mice and non-RA-6A2 antibody treated IFN- $\gamma$  treated mice were identical. Control experiments in vitro showed that a 44-fold excess of RA-6A2 antibody was administered over the amount required to neutralise the antiviral activity of the IFN- $\gamma$  administered (data not shown).

Figure 7.7

Effect of anti-IFN- $\gamma$  antibody on ability of IFN- $\gamma$  to increase the natural  
killer cell activity of C3H/He mouse splenic lymphocytes

Treatment	Effector : Target ratio		
	60:1	30:1	15:1
PBS + PBS	37 $\pm$ 2	32 $\pm$ 0	19 $\pm$ 1
E4-6A2 + IFN- $\gamma$	53 $\pm$ 2	40 $\pm$ 2	27 $\pm$ 1
PBS + IFN- $\gamma$	51 $\pm$ 3	42 $\pm$ 3	26 $\pm$ 2

- (i) 200  $\mu$ l of E4-6A2 antibody ascites fluid (neutralising titre  $10^{5.5}$ , see methods section I) or 200  $\mu$ l PBS were intraperitoneally administered to duplicate 9-week-old C3H/He mice. 24 hours later  $7 \times 10^3$  U IFN- $\gamma$  in 200  $\mu$ l PBS, or PBS alone, were intraperitoneally administered. After a further 19 hours spleens were removed and non-specific cytotoxic activity of lymphocytes tested in a 4 hour chromium release assay, with Yac-1 as target cells, as described in methods section M.
- (ii) Data are presented as mean  $\pm$  S.D. percentage lysis for duplicate mice.



### Discussion

The discussion has been split up into two sections, (a) Presence of IFN within the brains of SFV-infected C3H/He mice and effect on brain cell MHC antigen expression, (b) Preliminary studies using R4-6A2 anti-IFN- $\gamma$  monoclonal antibody to determine the role of IFN- $\gamma$  produced endogenously during SFV-infection of C3H/He mice.

#### (a) Presence of IFN within the brains of SFV-infected C3H/He mice and effect on brain cell MHC antigen expression

In this chapter the effect of avirulent A7(74) SFV infection of newborn and adult C3H/He mice on both the amount of IFN present within the brain and the level of MHC antigen expressed by cells within the brain is investigated. The data presented show that uninfected newborn and adult mice did not possess any detectable IFN activity within the brain. In contrast, SFV-infected newborn and adult mice clearly possessed IFN activity within the brain. The amount of IFN present within the brains of newborn mice, infected intraperitoneally with SFV, was found to increase throughout the course of infection (all mice died at day 2 post-infection) and correlated with an increase in the titre of virus present. The amount of IFN present within the brains of adult mice, infected intranasally with SFV, increased until around day 5 post-infection when maximal levels were reached, and thereafter declined to an undetectable level by day 10 post-infection. The IFN type present within the brains appeared to be IFN- $\alpha\beta$  since activity was stable at pH2 and was not significantly neutralised by monoclonal antibody to IFN- $\gamma$ . The possibility cannot be excluded that small amounts of IFN- $\gamma$  were indeed present and at a level too low to enable detection in the assay system used. Similarly, it

is possible that IFN- $\gamma$  present within the brains of SFV-infected mice may have been bound tightly to receptors for IFN- $\gamma$  on brain tissue and was not thereby detected in the brain extracts subjected to IFN assay. The fact that the increase in amount of IFN- $\alpha\beta$  present within the brains of newborn mice correlated with an increase in the of virus present suggests that the brain cells released IFN- $\alpha\beta$  when infected with SFV. Numerous studies by undergraduates at the Department of Biological Science, University of Warwick, have also demonstrated a correlation between the increase in amount of IFN- $\alpha\beta$  and increase in titre of virus present within the brains of adult Balb/c mice infected intranasally with either the virulent L10 strain or avirulent A7(74) strain of SFV. However, these observations differ from those reported previously by Oaten *et al.* (1976) and Fleming (1977) who did not find any correlation between the increase in amount of IFN- $\alpha\beta$  and increase in titre of virus present within the brains of adult Swiss A<sub>2</sub>C mice infected intracerebrally with the avirulent A7(74) strain of SFV and adult Porton mice infected intraperitoneally with either the virulent L10 strain or avirulent A7(74) strain of SFV. The difference in results obtained may be a reflection of the fact that different strains of mice and routes of inoculation with virus were used.

The effect of avirulent A7(74) SFV-infection on the level of class I (H-2K<sup>k</sup>) and class II (H-2A<sup>k</sup>) MHC antigen expressed by cells within the brains of newborn C3H/He mice was also investigated. The data presented show that none of the cells harvested from the brains of uninfected mice displayed any detectable H-2K<sup>k</sup> or H-2A<sup>k</sup> antigen on the cell-surface. These observations are consistent with a previous report by Wong *et al.* (1984) which demonstrated that less than 1% of cells harvested from the brains of uninfected newborn CBA mice displayed detectable class I or class II MHC antigen on the cell surface. In the present study, 6% of cells harvested

from the brains of SFV-infected mice were found to display H-2K<sup>k</sup> antigen on the cell surface, and 2% displayed H-2A<sup>k</sup> antigen. Hence, infection with SFV led to an increase in the number of cells which expressed class I and class II MHC antigen within the brains of newborn C3H/He mice. It is not possible from the data obtained to determine whether the cells which expressed H-2K<sup>k</sup> and H-2A<sup>k</sup> antigen were indeed brain cells or cells derived from blood. A future experimental approach which could be used to identify the cells would be to use a fluorescence activated cell sorter to sort the cells which express H-2K<sup>k</sup> and H-2A<sup>k</sup> antigen and then use a range of antibodies to stain markers specific for the possible cell types present (for further details see chapter 4 discussion). It does at least seem possible that some brain cells may have expressed H-2K<sup>k</sup> antigen since IFN- $\alpha$  was found to be present within the brains of SFV-infected newborn mice and IFN- $\alpha$  increases class I MHC antigen expression on brain cells (see chapter 4). Experiments in which brain extracts prepared from SFV-infected newborn mice were found to increase class I MHC antigen expression on astrocytes *in vitro* also indicate that IFN- $\alpha$  present within the brains had the ability to increase class I MHC antigen expression on brain cells. The fact that brain extracts prepared from SFV-infected newborn mice had no effect on class II MHC antigen expression by astrocytes *in vitro* also suggests that no IFN- $\gamma$  was present within the brains. It is however important to note that IFN- $\alpha$  inhibits the ability of IFN- $\gamma$  to induce class II MHC antigen expression on astrocytes (see chapter 4). Hence, large amounts of IFN- $\alpha$  present in the brain extracts may have inhibited the ability of any low levels of IFN- $\gamma$  also present to increase class II MHC antigen expression on the astrocytes. The observation that no class II MHC inducing activity was present in the brain extracts also suggests that the 2% of cells which expressed H-2A<sup>k</sup> antigen within the brains of SFV-infected newborn mice may have infiltrated into the brain from blood. A future

experimental approach to determine whether IFN- $\alpha\beta$  or IFN- $\gamma$  increased class I or class II MHC antigen expression on cells within the brains of SFV-infected newborn mice would be to intracerebrally administer anti-IFN- $\alpha\beta$  and anti-IFN- $\gamma$  antibody, prior to infection, and monitor the effect on the number of cells which express class I and class II MHC antigen within the brain.

Technical problems prevented a study of the effect of avirulent A7(74) SFV infection on the level of class I (H-2K<sup>k</sup>) and class II (H-2A<sup>k</sup>) MHC antigen expressed by cells within the brains of adult C3H/He mice. However, in vitro experiments demonstrated the ability of brain extracts prepared from SFV-infected adult mice to increase H-2K<sup>k</sup> antigen expression on astrocytes, indicating that class I MHC antigen expression may also be increased on cells within the brains of SFV-infected adult mice. Brain extracts prepared from mice at day 3, 5 and 7 post-infection increased H-2K<sup>k</sup> antigen expression on astrocytes to a level which correlated with the amount of IFN- $\alpha\beta$  present. In contrast, brain extracts prepared from mice at day 10 and 14 post-infection increased H-2K<sup>k</sup> antigen expression on astrocytes despite the absence of detectable IFN- $\alpha\beta$ . Since IFN- $\alpha\beta$  only increases H-2K<sup>k</sup> antigen expression on astrocytes at concentrations which may be detected by IFN assay (i.e. above 10 u/ml, see chapter 4 figure 4.13) these data suggest that IFN- $\alpha\beta$  may not have been the only factor present in the brain extracts which was able to increase class I MHC antigen expression on the astrocytes. It is possible that IFN- $\gamma$  may have been present in the brain extracts since IFN- $\gamma$  increases H-2K<sup>k</sup> antigen expression on astrocytes at concentrations which cannot be detected by IFN assay (i.e. at 0.01 to 1 u/ml, see chapter 4 figure 4.14). Very recently it has been shown that classical tumour necrosis factor (TNF- $\alpha$ ) also increases class I (but not class II) MHC antigen expression on astrocytes derived from C57 BL/6 and

Balb/c mice (Lavi *et al.*, 1988). This observation suggests the possibility that TNF- $\alpha$  may also have been present in the brain extracts prepared from both newborn and adult mice. A possible experimental approach to determine whether IFN- $\gamma$  or TNF- $\alpha$  were indeed present in the brain extracts would be to add anti-IFN- $\gamma$  or anti-TNF- $\alpha$  to the brain extracts, prior to addition to the astrocytes, and investigate the effect on the increase in class I MHC antigen expression. The observation that astrocytes treated with brain extracts prepared from the brains of SFV-infected newborn and adult mice were found to be more susceptible to alloreactive CTL lysis indicates that the factors present within the brains of infected mice were able to increase the ability of brain cells to participate in class I MHC restricted T-cell immune reactions. If a similar effect occurred *in vivo* during SFV-infection of mice the susceptibility of infected brain cells to lysis by virus-specific CTL would be increased thereby facilitating the clearance of virus from the brain.

The data presented also show that brain extracts prepared from SFV-infected adult mice had no effect on class II MHC antigen expression on astrocytes *in vitro*, indicating that class II MHC antigen expression may not be increased on cells within the brains of SFV-infected adult mice. However, the fact that brain extracts prepared from SFV-infected newborn mice also had no effect on class II MHC antigen expression on astrocytes *in vitro* and that 2% of cells within the brains of SFV-infected newborn mice were found to display class II MHC antigen does at least indicate that some cells which express class II MHC antigen may also be present within the brains of SFV-infected adult mice. Cells which express class II MHC antigen within the brains of SFV-infected mice may present viral antigen to T-helper cells and thereby play a role in the amplification of the immune response within the brain. IFN- $\alpha\beta$  present within the brains of SFV-infected mice would

inhibit the replication of SFV in brain cells (see chapter 4 figures 4.1, 4.3 and 4.5) and possibly contain the spread of virus within the brain until an immune response developed.

(b) Preliminary studies using R4-6A2 anti-IFN- $\gamma$  monoclonal antibody to determine the role of IFN- $\gamma$  produced endogenously during SFV-infection of C3H/He mice

It was reasoned that if IFN- $\gamma$  produced endogenously in an SFV-infected mouse functions in the generation or expression of an immune response, then the administration of an anti-IFN- $\gamma$  monoclonal antibody, to block IFN- $\gamma$  mediated function(s), would result in an exacerbation of disease. Adult C3H/He mice were therefore treated with R4-6A2 rat-anti-murine IFN- $\gamma$  monoclonal antibody, prior to infection with 1LD<sub>50</sub> of the avirulent A7(74) strain of SFV, and the effect on the clinical course of SFV disease studied. The data presented show that intraperitoneal treatment with R4-6A2 antibody had no clear effect on either the clinical course of SFV disease or the number of mice surviving infection. Since the titre of R4-6A2 antibody present in the serum of treated mice was found to remain at a high level throughout the time-course over which the clinical course of SFV disease was monitored, these observations suggest that either endogenously produced IFN- $\gamma$  did not have any role in controlling infection by SFV, or the R4-6A2 antibody did not neutralise the activity of IFN- $\gamma$  *in vivo*. The ability of R4-6A2 antibody to block the induction by exogenous recombinant IFN- $\gamma$  of natural killer (NK) cell activity in mouse splenic lymphocytes was thereby investigated in order to determine whether R4-6A2 antibody had the ability to neutralise the activity of IFN- $\gamma$  *in vivo*. The data presented show that intraperitoneal treatment with R4-6A2 antibody did not block the ability of intraperitoneally administered recombinant IFN- $\gamma$  to increase the

NK cell activity of mouse splenic lymphocytes, despite the fact that mice were treated with a 44-fold excess of antibody over the amount required in vitro to neutralise the antiviral activity of the IFN- $\gamma$  administered. Recent studies by Mr R Darley (University of Warwick) have shown that co-administration of IFN- $\gamma$  with a 60 fold-excess of E4-6A2 antibody slightly reduced the ability of IFN- $\gamma$  to increase the NK cell activity of splenic lymphocytes (unpublished observations). These observations suggest that the E4-6A2 antibody is able to neutralise the ability of IFN- $\gamma$  to increase the NK cell activity of splenic lymphocytes in vivo, but only when present in large excess (in terms of the amount required to neutralise antiviral activity in vitro). Whether the E4-6A2 antibody neutralises the antiviral activity of IFN- $\gamma$  in vivo, and whether similar amounts of antibody are required as in vitro, remains to be determined.

Schreiber et al. (1985) generated 4 monoclonal hybridomas (H1, H2, H21, H22) that produced anti-murine IFN- $\gamma$  monoclonal antibody. They found that two of the antibodies (H1 and H2) had a specificity for an epitope on the amino terminus of IFN- $\gamma$  and two (H21 and H22) had a specificity for an epitope on the carboxyl terminus. All of the antibodies inhibited the antiviral activity of IFN- $\gamma$  but only the antibodies directed against the carboxyl terminal epitope of IFN- $\gamma$  inhibited the macrophage activating activity (for tumour cytotoxicity) of IFN- $\gamma$ . These studies suggest that there are at least two functional domains on murine IFN- $\gamma$ . While both of the domains appear to be involved in the antiviral activity of IFN- $\gamma$ , it appears that only one of the domains plays a role in the macrophage activating activity of IFN- $\gamma$ . Studies by Langford et al. (1983) have also shown that polyclonal antibodies directed to a synthetic peptide corresponding to 20 amino-acids of the amino terminus of murine IFN- $\gamma$  abrogated the antiviral activity of IFN- $\gamma$ . Similarly, Gray and

Rindarknacht observed that IFN- $\gamma$  lacking 18 amino-acids of the carboxyl terminus displayed less than 1% of the antiviral activity of complete IFN- $\gamma$  (reported by Schreiber *et al.*, 1985). Taken together, these studies also indicate that both amino and carboxyl termini are important for the expression of IFN- $\gamma$  antiviral activity.

Since IFN- $\gamma$  contains distinct functional domains responsible for different activities, it is possible that in the present study the 44-fold excess of R4-6A2 monoclonal antibody (in terms of the amount required to neutralise antiviral activity *in vitro*) did not neutralise the ability of IFN- $\gamma$  to increase the NK cell activity of splenic lymphocytes *in vivo* because the epitope recognised by the R4-6A2 antibody was on a different domain on IFN- $\gamma$  to that which is responsible for mediating the NK cell activity of IFN- $\gamma$ . The epitope on IFN- $\gamma$  recognised by the R4-6A2 monoclonal antibody remains to be determined. Previous studies by Vogel *et al.* (1986) have demonstrated that IFN- $\gamma$  induces an antiviral state, Fc receptor expression and class II MHC antigen expression on macrophages *in vitro* over the same dose-response curve. However, 10 to 30 times more R4-6A2 monoclonal antibody was required to inhibit the induction by IFN- $\gamma$  of Fc receptor expression and an antiviral state that was required to inhibit induction of class II MHC antigen expression. These observations suggest that the R4-6A2 monoclonal antibody recognises an epitope on a domain of IFN- $\gamma$  responsible for mediating class II MHC antigen inducing activity and which may be distinct from the two domains which mediate antiviral activity.

Taken, together the results of these studies emphasize the point that extreme caution must be exercised in interpreting findings in which a given anti-IFN- $\gamma$  monoclonal antibody is either found to be ineffective or only partially effective in abolishing an effect believed to be mediated by



IFN- $\gamma$ . The amount of antibody present and whether the epitope on the IFN- $\gamma$  molecule recognised by the antibody has a role in the expression of an IFN- $\gamma$  mediated activity must be given consideration prior to reaching a conclusion as to whether IFN- $\gamma$  mediates an activity, based on the degree of neutralisation achieved with the monoclonal antibody. It is possible that in the present study treatment of mice with R4-6A2 monoclonal antibody, prior to infection with SFV, had no clear effect on either the clinical course of SFV disease or number of mice surviving infection because the R4-6A2 monoclonal antibody (under the conditions used) did not neutralise the activities of IFN- $\gamma$  which are responsible for controlling infection by SFV. A comprehensive study of the ability of R4-6A2 monoclonal antibody to neutralise the various activities of exogenous IFN- $\gamma$  *in vivo* would define the activities of IFN- $\gamma$  which are neutralised by the R4-6A2 monoclonal antibody. The antibody could then be used in studies on the role of IFN- $\gamma$  produced endogenously during SFV-infection of mice with the knowledge of which activities of IFN- $\gamma$  may be neutralised. The use of a range of anti-IFN- $\gamma$  monoclonal antibodies to different functional domains of IFN- $\gamma$  (hence which neutralise different activities of IFN- $\gamma$ ) would also provide information about the roles of the different activities of IFN- $\gamma$  during infection of mice with SFV. A polyclonal antiserum to recombinant IFN- $\gamma$  (recombinant IFN- $\gamma$  to exclude the possibility of contamination with other lymphokines which may be present in natural IFN- $\gamma$  preparations) could be used to study the 'overall' role of IFN- $\gamma$  produced endogenously during infection of mice with SFV. To determine the role of IFN- $\gamma$  produced endogenously during SFV-infection of mice a number of parameters may be studied including: time course of development of clinical symptoms of SFV disease and death; number of mice surviving infection; number of mice surviving infection which are immune to further infection by lethal doses of SFV; titre of IFN- $\alpha\beta$  and SFV present in the brain, serum and spleen;

titre of anti-SFV neutralising and cytolytic antibody (both IgM and IgG) in serum and brain: development of SFV-specific cytotoxic T-cells: ability of T-cells to release IFN- $\gamma$  in response to SFV antigen presented by accessory cells: level of class I and class II MHC antigen expression on both brain cells and cells present in blood.

Since the blood brain barrier normally prevents the entry of antibody into the brain (Wekerle *et al.*, 1986b) it is possible that intraperitoneally and intravenously administered anti-IFN- $\gamma$  antibody may not enter the brain. Hence, even if anti-IFN- $\gamma$  antibody had the ability to neutralise all activities of IFN- $\gamma$  *in vivo* it may not reach sites of IFN- $\gamma$  production within an SFV infected mouse brain. In the present study, brain extracts prepared from uninfected mice treated intraperitoneally with R4-6A2 monoclonal antibody were found to contain very low titres of IFN- $\gamma$  neutralising activity. However, these low titres of IFN- $\gamma$  neutralising activity may be accounted for by the presence of R4-6A2 antibody in serum within the capillaries of the brain rather than by R4-6A2 antibody actually crossing the blood brain barrier and entering the brain. Since the blood brain barrier also normally prevents the entry of T-cells into the brain (Wekerle *et al.*, 1986b), and IFN- $\gamma$  is produced exclusively by activated T-cells, the question arises as to whether IFN- $\gamma$  would ever be produced within a brain which has an intact blood brain barrier. This does seem likely since activated T-cells (but not resting T-cells) have been shown to attach to endothelial cells and subsequently penetrate the barrier (possibly via the release of endoglycosidase, Naparstek *et al.*, 1983, 1984). It is possible that during acute SFV-encephalitis the blood brain barrier may break down thereby allowing anti-IFN- $\gamma$  to enter the brain. Future experiments in which anti-IFN- $\gamma$  is intracerebrally administered to mice, prior to infection with SFV, may provide an insight into the role of IFN- $\gamma$  (if it is present) within the brain.

## Chapter 8

### Final discussion

The aims of this thesis were to investigate the ways in which IFN treatment may modulate the ability of SFV-infected brain cells to participate in T-cell mediated immune reactions, and to study the role of IFN- $\gamma$  produced endogenously during SFV-infection of mice via administration of a neutralising monoclonal antibody to IFN- $\gamma$ . In this final chapter, the major points of the thesis will be interrelated, and the possible significance during SFV-infection of mice discussed. The possible significance of these studies to the understanding of the roles of IFNs in human disease will also be discussed.

As mentioned in chapter 1, SFV exists in essentially two forms, avirulent and virulent (wild type). In adult mice, the avirulent form produces a self-limiting demyelinating disease which is usually subclinical, whereas the virulent form causes a rapid lethal encephalitis. The role of T-lymphocytes in recovery from avirulent SFV-infection has been studied using nude (nu/nu; T-cell deficient) mice, and by analysing the activity of T-lymphocytes from infected mice (Atkins *et al.*, 1985). Jagelman *et al.* (1978) showed that the brains of nude mice, infected with avirulent SFV, did not have focal demyelinating lesions, whereas lesions were present in infected phenotypically normal (nu/+) control mice. Furthermore, clearance of virus from the brains of avirulent SFV-infected nude mice was both delayed and incomplete (Jagelman *et al.*, 1978). Bradish *et al.* (1979) and Fazakerley *et al.* (1983) confirmed that clearance of virus from the brains of avirulent SFV-infected nude mice was incomplete, and showed that T-cell dependant IgG neutralising antibody synthesis and efficient clearance of

virus could be restored by the transfer of spleen cells from immune competent phenotypically normal (nu/+) control mice. Fleming (1977) also showed that the appearance of T-cell dependent (IgG) neutralising antibody within the brains of avirulent SFV-infected Porton mice correlated with a decline in the titre of virus in the brain, and suggested that the antibody may be involved in the clearance of virus from the brain. Reconstitution of avirulent SFV-infected nude mice with purified T-cells from SFV sensitized mice, or with spleen cells from SFV sensitized and unsensitized mice, also resulted in the formation of demyelinating lesions in the brain (Fazakerley *et al.*, 1983, 1987). In contrast, no demyelinating lesions were found in the brains of avirulent SFV-infected nude mice reconstituted with SFV-sensitized spleen cells depleted of T-cells (Fazakerley *et al.*, 1987). Taken together, the results of these studies clearly indicate a role for T-cells in both the clearance of virus and formation of demyelinating lesions. It is however important to note that T-cells may not be the only component of the immune system involved in the formation of demyelinating lesions. Thus, Berger (1980) carried out reconstitution experiments following immune suppression of avirulent SFV-infected CBA mice with cyclophosphamide and showed that SFV-sensitized spleen cells depleted of T-cells could reconstitute lesions, as could spleen cell populations depleted of B-cells. When both T and B cells were depleted, no lesions developed, and lesions were most severe when both T and B cells were present.

Since the blood brain barrier normally prevents the entry of T-cells into the brain, and brain cells do not usually express MHC antigens (which are required for T-cell recognition of antigen), the question arises as to how T-cells may be involved in the clearance of virus and formation of demyelinating lesions within an avirulent SFV-infected mouse brain. Based

upon the observations made in this thesis, and previous studies by other workers, it is possible to present a hypothetical model for the mechanism whereby T-cells participate in the immune response during SFV-infection of mice.

Intraperitoneal infection of adult mice with the avirulent strain of SFV results in viraemia which is accompanied by an increase in the titre of virus present in the spleen, and is followed by an increase in the titre of virus present in the brain (Bradish and Allner, 1972). IFN- $\alpha\beta$  present in the serum and brain early in infection may play a role in controlling viral replication to some extent, as shown by experiments in which administration of anti-IFN- $\alpha\beta$  serum to SFV-infected mice was found to result in increased virus titres in the brain and increased mortality (Fauconnier, 1970, 1971). SFV present in the spleen may be processed by macrophages (and possibly also by dendritic cells in lymph nodes) and presented to SFV-specific T-helper cells leading to the initiation of the SFV-specific T-cell response. The clearance of viraemia is associated with an increase in T-cell independent (IgM) anti-SFV neutralising antibody in serum at around day 4 post-infection (Fleming, 1977; Bradish *et al.*, 1979), and may also be mediated to some extent by natural killer cells (possibly activated by IFN- $\alpha\beta$  in serum: Macfarlan *et al.*, 1977; Djeu *et al.*, 1979). The increase in T-cell independent (IgM) anti-SFV neutralising antibody in serum is not associated with a reduction in the titre of virus in the brain, possibly due to the inability of IgM to cross the blood brain barrier (Fleming, 1977; Bradish *et al.*, 1979). SFV-specific CTL activity in the spleen reaches a detectable level at around day 5 post-infection, and is maximal around day 7 post-infection (Blackman and Morris, 1984). The ability of spleen cells to release IFN- $\gamma$  in response to SFV antigen also develops in parallel to SFV-specific CTL activity (Blackman and Morris, 1984). It is

possible that SFV-specific CTL may also play a role in the final clearance of SFV-infected cells from sites outside the brain. Furthermore, IFN- $\alpha\beta$  present in serum early in infection may increase class I MHC antigen expression on cells at a number of sites outside the brain and hence increase the recognition of SFV-infected cells by SFV-specific CTL. T-cell dependant (IgG) anti-SFV antibody is secreted from about day 5 post-infection, and is the predominant form of antibody present in serum at day 7 post-infection (Fleming, 1977). The appearance of T-cell dependant (IgG) antibody in the brain at around day 5 post-infection correlates with a rapid decline in the titre of virus (Fleming, 1977). The appearance of IgG antibody in the brain may not however reflect a general breakdown of the blood brain barrier since the subclasses detected were IgG<sub>2a</sub> and IgG<sub>3</sub>, which are reported to permeate membranes with great facility (Fleming, 1977). Interleukin-1 (IL-1: T-cell activation factor) and IFN- $\gamma$  released into serum during the T-cell activation process would increase intercellular adhesion molecule-1 (ICAM-1) expression on endothelial cells (Dustin *et al.*, 1986) and thereby facilitate the binding of SFV-specific T-cells bearing lymphocyte function associated antigen-1 (LFA-1, the receptor for ICAM-1: Springer *et al.*, 1987). McCarron *et al.* (1985) showed that treatment of brain endothelial cells with concanavalin-A conditioned medium (which contains IFN- $\gamma$ ) resulted in the induction of class II MHC antigen expression and ability to present guinea pig basic protein to sensitized lymph node cell cultures depleted of macrophages-monocytes. IFN- $\gamma$  present in the serum of an SFV-infected mouse may thereby also induce class II MHC antigen expression on brain endothelial cells and enable the cells to present SFV antigen to SFV-specific T-helper cells leading to the further amplification of the T-cell response. Activated SFV-specific T-cells bound to brain endothelial cells may then penetrate the blood brain barrier via the release of endoglycosidases (Naparstek *et al.*, 1983, 1984). Having

arrived in the brain, SFV-specific CTL would be able to recognise SFV antigen on astrocytes, oligodendrocytes and neurons since IFN- $\alpha\beta$  produced in the brain early in infection would have induced class I MHC antigen expression on the cells. SFV-specific CTL may thereby lyse SFV-infected brain cells and hence play a role in the clearance of virus from the brain. Furthermore, IFN- $\gamma$  released by SFV-specific CTL on recognition of SFV antigen on brain cells may induce class II MHC antigen expression on astrocytes and enable the cells to present SFV antigen to SFV-specific T-helper cells within the brain. Frei *et al.* (1987) showed that recombinant IFN- $\gamma$  treatment of murine microglial cells resulted in the induction of class II MHC antigen expression and ability to present ovalbumin to ovalbumin-specific T-helper cells. IFN- $\gamma$  released by SFV-specific CTL on recognition of SFV antigen on brain cells may thereby also induce class II MHC antigen expression on microglial cells and enable the cells to present SFV antigen to SFV-specific T-helper cells. Both astrocytes and microglial cells have been shown to release IL-1 (Fontana *et al.*, 1982; Giulian *et al.*, 1986). The significance of intracerebral IL-1 synthesis may be fundamental to further T-cell activation within the brain since IL-1 enhances T-cell production of Interleukin-2 (IL-2, T-cell growth factor) and the expression of IL-2 receptors on T-cells (Fontana *et al.*, 1987). It is possible that IFN- $\gamma$  released by SFV-specific CTL on recognition of SFV antigen on brain cells may not actually increase class II MHC antigen expression on astrocytes and microglial cells until the amount of IFN- $\alpha\beta$  within the brain declines at around day 7 post-infection, since IFN- $\alpha\beta$  was shown to inhibit the ability of IFN- $\gamma$  to induce class II MHC antigen expression on brain cells. Astrocytes have also been shown to release interleukin-3 (IL-3, multiple colony stimulating factor, mast cell growth factor, F [persistent]-cell stimulating factor; Frei *et al.*, 1985), prostaglandin E (PGE; Fontana *et al.*, 1982), and tumour necrosis factor

(TNF, whether  $\alpha$  or  $\beta$  not specified: Robbins *et al.*, 1987). Microglial cells also release TNF- $\alpha$  (Frei *et al.*, 1987). PGE and IL-3 have been shown to inhibit the ability of IFN- $\gamma$  to induce class II MHC antigen expression on macrophages and mast cells, respectively (Snyder *et al.*, 1982; Wong *et al.*, 1984b). Whether PGE and IL-3 inhibit the ability of IFN- $\gamma$  to induce class II MHC antigen expression on astrocytes and microglial cells remains to be determined. However, if this was indeed the case, IL-3 and PGE released by astrocytes during the inflammatory processes within the brain may also represent an important negative feedback mechanism during the T-cell activation process. IFN- $\gamma$  released by SFV-specific CTL and helper T-cells on recognition of SFV antigen on brain cells, and TNF released by astrocytes and microglial cells, would also increase class I MHC antigen expression on astrocytes (Lavi *et al.*, 1988), as well as on oligodendrocytes, microglial cells and neurons for the case for IFN- $\gamma$  (Wong *et al.*, 1984), hence increase the susceptibility of the cells to SFV-specific CTL lysis. IFN- $\gamma$  would increase class I MHC antigen expression in the presence of IFN- $\alpha\beta$ , at least on astrocytes, since IFN- $\alpha\beta$  and IFN- $\gamma$  were shown to have an additive effect with regard to the augmentation of class I MHC antigen expression. Interleukin-1 has been shown to stimulate the proliferation of astrocytes (but not oligodendrocytes) (Giulian and Lachman, 1985). Similarly, IL-3 induces the growth of microglial cells (Frei *et al.*, 1986). IL-1 and IL-3 present in an SFV-infected brain may thus stimulate the proliferation of astrocytes and microglial cells, and thereby essentially replace those cells lysed by the virus or by SFV-specific CTL. This would also ensure the maintenance of a high number of cells which may be induced to express class II MHC antigen, hence which may present SFV antigen to SFV-specific T-helper cells within the brain. As mentioned above, the appearance of IgG antibody in the brains of SFV-infected mice at around day 5 post-infection correlates with a rapid



decline in the titre of virus. Since untreated microglial cells have been shown to express Fc receptors and to possess a phagocytic activity (Frai *et al.*, 1987) it does at least seem a strong possibility that microglial cells may play a role in the clearance of virus from the brain. The activities of T-cell dependent anti-SFV IgG neutralising antibody, IFNs- $\alpha\beta$  and - $\gamma$ , IL-1, -2 and -3, TNF, astrocytes, microglial cells, SFV-specific CTL and T-helper cells may thereby account for the clearance of virus from the brains of avirulent SFV-infected mice, at around day 10 post-infection (Jagelman *et al.*, 1978).

A possible experimental approach to determine whether SFV-specific CTL actually play a role in the clearance of virus from the brains of SFV-infected mice would be to administer anti-Lyt 2 antibody to mice prior to infection with SFV. Since class I MHC restricted CTL express the Lyt 2 antigen (Male *et al.*, 1987) this procedure would ensure the depletion of class I MHC restricted SFV-specific CTL generated in response to SFV-infection and allow comparison of the titres of virus in the brain with and without the presence of class I MHC restricted SFV-specific CTL. Similarly, the role of SFV-specific T-helper cells in the production of T-cell dependent IgG antibody and the generation of SFV-specific CTL could be studied via the administration of anti-L3T4 antibody to mice, prior to infection with SFV, since this procedure would ensure the depletion of SFV-specific T-helper cells which express the L3T4 antigen (and also class II MHC restricted SFV-specific CTL which express L3T4 antigen: Male *et al.*, 1987). The experimental protocol used may be based on that by Ranges *et al.* (1987) who used anti-L3T4 monoclonal antibody to study the role of L3T4<sup>+</sup> cells in humoral and cell-mediated responses to tetanus toxoid. The role of IFN- $\alpha\beta$ , IFN- $\gamma$ , TNF and IL-3 in the induction or inhibition of class I and class II MHC antigens on cells within the brains of SFV-infected mice

may be studied by intracerebral administration of neutralising antibody to each of these cytokines, prior to infection with SFV, and subsequent analysis of brain cell MHC antigen expression. The fact that intracerebral administration will damage the blood brain barrier must however be considered when interpreting the results of such a study.

Jagelman *et al.* (1978) reported that demyelination first began at the time just after virus had been cleared from the brain. Demyelination may be an indirect result of the release of myelin antigens brought about by lysis of oligodendrocytes, the myelin producing cell in the brain (Atkins *et al.*, 1985). Thus, lysis of oligodendrocytes due to the direct cytopathic effect of SFV (Atkins and Sheahan, 1982; Atkins, 1983), the activity of SFV-specific CTL, or the presence of TNF (Robbins *et al.*, 1987), may result in the release of myelin antigens which are normally sequestered from the immune system. Astrocytes, endothelial cells, and possibly microglial cells, expressing class II MHC antigens may then present myelin antigen to myelin-specific T-helper cells (Fontana *et al.*, 1984; Fierz *et al.*, 1985), and T-cells thus sensitized could mount a demyelinating autoimmune attack on the brain (Zamvil *et al.*, 1985). Since only activated T-cells appear to cross the blood brain barrier (Naparstek *et al.*, 1983, 1984; Wekerle *et al.*, 1986), non-activated myelin specific T-helper cells may only enter the brain and hence recognise myelin presented by astrocytes and microglial cells after a breakdown of the blood brain barrier due to SFV infection. Evidence that astrocytes may indeed be involved in the formation of demyelinating lesions has been obtained by light and electron microscopic studies of sections prepared from the brains of SFV-infected mice. Thus, Kelly *et al.* (1982) reported that astrocyte processes were expanded in most demyelinating areas, and Pathak *et al.* (1983) reported that astrocytes could be easily seen in lesion areas, particularly surrounding demyelinated

axons. Furthermore, the development of demyelinating lesions was shown to be accompanied by an infiltration of lymphocytes possessing characteristic projections which extended deep into the cytoplasm of adjacent astrocytes (Kelly *et al.*, 1982; Pathak *et al.*, 1983). Macrophages were also found to be present in the lesion areas and again projections of lymphoblastic cells were seen projecting into the cytoplasm of these cells without any obvious damage being caused (Pathak *et al.*, 1983). Degenerated myelin was also observed in both astrocytes and macrophages (Pathak *et al.*, 1983). The origin of the macrophages observed was not clear although Pathak *et al.* (1983) suggested that at least the majority of macrophages may have infiltrated into the brain from blood, whilst some may be derived from activated microglial cells. It is tempting to speculate that the astrocytes, macrophages, and at least some activated microglial cells may have been presenting myelin to myelin-specific T-cells around these demyelinating lesions. It would be interesting to determine in future experiments whether the astrocytes and macrophages which interacted with lymphocytes around the demyelinating lesions actually expressed class I and class II MHC antigens. Similarly, it would be of interest to determine the actual identity of the lymphocytes which interacted with the astrocytes and macrophages. This may be achieved by the indirect immunochemical staining of brain sections with antibody to Lyt 2, L3T4, class I MHC antigen or class II MHC antigen and visualisation by light microscopy. Furthermore, the importance of class II MHC antigen expression in the development of demyelinating lesions could be investigated by studying the effect of intracerebral administration of anti-class II MHC antigen antibody. It would also be interesting to determine whether intracerebral administration of anti-IFN- $\gamma$  antibody prevents the induction of class II MHC antigen expression on brain cells and the development of demyelinating lesions.

Another experimental model of demyelination in mice and rats, experimental allergic encephalomyelitis (EAE), does not involve virus infection, but is induced by the injection of myelin together with adjuvants (Craggs and Webster, 1985). The formation of lesions in EAE has also been shown to be mediated by an autoimmune T-cell reaction directed against myelin basic protein (MBP) (for review see Wekerle *et al.*, 1986b). Furthermore, it has been shown that class II MHC antigen expression is dramatically increased around lesions of EAE in rat brains (Craggs and Webster, 1985; Vass *et al.*, 1986). Craggs and Webster (1985) and Vass *et al.* (1986) both identified macrophages which expressed class II MHC antigen around lesions. Craggs and Webster (1985) also suggested that some class II MHC antigen positive astrocytes may have been present, while Vass *et al.* (1986) reported that activated microglial cells expressed class II MHC antigen and astrocytes did not. In both studies, the cells expressing class II MHC antigen were proposed to have a role in antigen presentation which led to the development of EAE lesions.

The results of these studies may assist in the understanding of the pathogenesis of the most common demyelinating disease of man, multiple sclerosis, which has been proposed to have a viral aetiology (McKhann, 1982). Traugott *et al.* (1983) showed that the progression of demyelinating lesions in multiple sclerosis was associated with the presence of large numbers of T-helper and cytotoxic T-cells around the lesion margin, and that class II MHC antigen positive macrophages were present within lesions. Subsequent studies also demonstrated both class I and class II MHC antigens on astrocytes and endothelial cells, as well as class II MHC antigen on macrophages (Traugott and Raine, 1985; Traugott, 1987). While class I and class II MHC antigen positive astrocytes were mainly found at the edge of active lesions, endothelial cells expressing class I and class II MHC

antigen were found to be randomly distributed throughout the brain (Traugott and Raine, 1985; Traugott, 1987). These observations suggest that class II MHC antigen positive astrocytes and macrophages may present myelin to T-helper cells during lesion development. Class II MHC antigen positive endothelial cells may also present myelin to T-helper cells in blood and thereby amplify the autoimmune T-cell response. Myelin-specific class I restricted CTL may lyse class I MHC antigen positive astrocytes (and oligodendrocytes) which have bound fragments of myelin, and hence play a role in the formation of lesions. The factor(s) involved in the initiation of a T-cell autoimmune response and the induction of class I and class II MHC antigen expression on brain cells in multiple sclerosis are not yet known. The results of these studies do however at least suggest a possible mechanism. Thus, some virus infection of the brain (possibly subclinical) may result in the release of IFN- $\alpha\beta$  which increases class I MHC antigen expression on brain cells. Activated infiltrating virus-specific CTL may then lyse virus-infected brain cells, and release IFN- $\gamma$  on recognition of viral antigen. IFN- $\gamma$  thus released would increase class I and class II MHC antigen expression on astrocytes, microglial cells and infiltrating macrophages. Oligodendrocyte lysis due to either the cytopathic effect of the virus, the presence of TNF, or the activity of virus specific CTL may result in the release of myelin which may then be presented to infiltrating myelin specific T-helper cells by class II MHC antigen positive astrocytes, microglial cells or macrophages leading to the initiation of the myelin-specific autoimmune T-cell response.

Panitch et al. (1987) recently reported that intravenous administration of recombinant IFN- $\gamma$  resulted in an increase in the number of circulating monocytes which expressed class II MHC antigen, and led to an exacerbation of multiple sclerosis. It is possible that the exacerbation of disease may

have been due to an increase in the number of class II MHC antigen positive macrophages which infiltrated into the brain and subsequently played a role in lesion development. In contrast, intracerebral administration of natural IFN- $\beta$  has been shown to reduce exacerbations of multiple sclerosis (Jacobs *et al.*, 1981; Jacobs *et al.*, 1986), possibly due to the ability of IFN- $\beta$  to inhibit the induction by IFN- $\gamma$  of class II MHC antigen expression on brain cells. It may be therapeutically important to determine whether intracerebral administration of anti-IFN- $\gamma$  or anti-class II MHC antigen antibody reduce exacerbations of multiple sclerosis.

The results of these studies also suggest a possible mechanism whereby IFNs may play a role in the immune response to viral infection of the human brain. Thus, IFN- $\alpha\beta$  produced by brain cells in response to virus infection may help to contain the spread of virus until the cellular and humoral immune responses develop. Furthermore, IFN- $\alpha\beta$  would also increase class I MHC antigen expression on brain cells, and thereby increase recognition of brain cells by infiltrating virus-specific CTL. Virus-specific CTL may then lyse virus-infected brain cells, and also release IFN- $\gamma$  on recognition of viral antigen. IFN- $\gamma$  thus released would increase class II MHC antigen expression on astrocytes, microglial cells and infiltrating macrophages, and thereby enable these cells to present viral antigen to T-helper cells within the brain. Evidence that IFN may indeed be present in the brains of humans with viral encephalitis was obtained by Luby *et al.* (1969) who showed that IFN was present within the brains of patients who were dying from St Louis encephalitis virus (the class of IFN detected was not specified and the assay procedure used would have enabled the detection of both IFN- $\alpha\beta$  and IFN- $\gamma$ ).

Further experiments on the interactions between brain cells and components

of the immune system may provide a clearer understanding of the roles of the immune system in both resistance to viral disease and the pathogenesis of autoimmune disease. It may then be possible to develop strategies of immunomodulation for therapeutic purposes.

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**Key words:** SFV; interferon ( $\text{MolFN-}\alpha\beta$ ); MHC antigen expression; CTL.

### Infection of Cultured Murine Brain Cells by Semliki Forest Virus: Effects of Interferon- $\alpha\beta$ on Viral Replication, Viral Antigen Display, Major Histocompatibility Complex Antigen Display and Lysis by Cytotoxic T Lymphocytes

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#### SUMMARY

Primary brain cell cultures prepared from newborn mice were infected with Semliki Forest virus (SFV). The effects of interferon (IFN- $\alpha\beta$ ) treatment on SFV replication, SFV and major histocompatibility complex (MHC) class I antigen expression, and susceptibility to lysis by SFV-specific cytotoxic T lymphocytes (CTL) were determined. The IFN- $\alpha\beta$  treatment prevented replication of SFV as determined by incorporation of [<sup>3</sup>H]uridine into SFV RNA and very markedly reduced the expression of SFV antigens on the cell surface as determined by lysis with antibody and complement or indirect immunofluorescence. However, IFN- $\alpha\beta$  increased expression of MHC class I antigens, measured by indirect immunofluorescence and as assessed indirectly by susceptibility to killing by alloreactive T cell lines. SFV infection had no effect on MHC class I expression in either IFN- $\alpha\beta$ -treated or -untreated cells. The infected IFN- $\alpha\beta$ -untreated brain cells were susceptible to killing by the CTL at effector/target ratios in the range 3 to 30. The killing was MHC antigen-restricted, and uninfected cells were not killed. A target cell (YAC) highly susceptible to natural killer cell cytotoxicity was not killed by the CTL. IFN- $\alpha\beta$  treatment prior to SFV infection resulted in an augmentation of lysis by the CTL, indicating that even where SFV antigen expression is reduced, in the context of enhanced MHC class I expression brain cells remain susceptible to CTL killing.

#### INTRODUCTION

Infection of mice with virulent strains of Semliki Forest virus (SFV) which replicates to very high titres in the central nervous system (CNS) results in a severe encephalitis and death of the mice (Atkins *et al.*, 1985). The mechanism of the encephalitis is unclear; it may be due either to direct cytopathic effects of the virus replication in brain cells (Gates *et al.*, 1985) or to immune-mediated damage to infected cells. Evidence from reconstitution of SFV-infected nude mice with normal spleen cells (Fazakerley *et al.*, 1983) and adoptive transfer of SFV-sensitized spleen cells (Berger, 1980) suggests a contribution of cell-mediated immunity to the pathological process.

There are several ways in which cell-mediated immunity might result in encephalitis. One of the most obvious is that SFV-specific cytotoxic T-lymphocytes (CTL) infiltrating into the infected brain could lyse infected brain cells. However, for CTL lysis to occur, the target cell must bear not only 'foreign' (in this case viral) antigen but also self major histocompatibility complex (MHC) class I antigens (Zinkernagel & Doherty, 1974, 1978). Brain cells normally express very little MHC class I antigens (Viretta & Capra, 1978) and hence should be relatively insensitive to CTL lysis. However, during SFV infection large amounts of interferon (IFN- $\alpha\beta$ )

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may be present in the brain (A. G. Morris & N. J. Dimmock, unpublished observations); IFN- $\alpha$  is known to stimulate MHC antigen expression in a range of cell types, including brain cells (Wong *et al.*, 1983). Therefore an obvious possibility is that IFN- $\alpha$  produced during infection of mice by SFV stimulates MHC antigen expression in infected brain cells, thus making them more susceptible to lysis by CTL. We have previously shown that IFN- $\alpha$  treated SFV-infected fibroblasts or lymphoblastoid cells (which normally express MHC antigens) remain susceptible to CTL despite complete inhibition of SFV replication in these cells (Blackman & Morris, 1985). We have now extended these observations to primary brain cell cultures derived from newborn mice in order to study whether this occurs in SFV's major target cells. We find that IFN- $\alpha$  treatment of these cells induces raised expression of MHC class I antigen; SFV replication is then blocked and the display of SFV antigens markedly reduced; however, CTL lysis is augmented.

#### METHODS

**Viruses and cells.** The avirulent strain of SFV (AT77; Bradish *et al.*, 1971) was used for immunization of mice and the virulent L10 strain for *in vivo* infection of target cells. Stocks of these viruses were prepared and titrated as previously described (Blackman & Morris, 1984).

L29 and C3H10T $\frac{1}{2}$  (Krasznai *et al.*, 1973) fibroblasts were cultured in the Glasgow modification of Eagle's MEM (GMEM; Gibco) supplemented with 10% newborn calf serum (Gibco). Lymphoblastoid cells EL4 (H-2<sup>b</sup>), RDM4 (H-2<sup>d</sup>) and YAC (a natural killer cell target) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% foetal calf serum (Gibco).

Primary brain cell cultures were prepared from 1- or 2-day-old C3H-Ha or DBA mice (obtained from Olac, Bicester, U.K.) which are H-2<sup>b</sup> and H-2<sup>k</sup> respectively. Brains were removed from the mice, meninges separated by rolling on sterile filter paper and the tissues were disaggregated mechanically. The tissues were then reduced to single-cell suspensions by trypsinization. Sometimes deoxyribonuclease (Sigma) was used (at 20  $\mu$ g/ml for 30 min at 37°C) to reduce viscosity due to released DNA. The resulting cell suspension was pelleted at low speed, washed and then repelleted and resuspended in Dulbecco's modification of MEM (Gibco) supplemented with 10% foetal calf serum. They were then planted in 5 cm Petri dishes (Nunc, Roskilde, Denmark) at  $2 \times 10^6$  cells per dish. Cells grew rapidly initially and were generally nearly confluent at 5 to 7 days after seeding, at which time they were used for experiments. Indirect immunofluorescence microscopy of permeabilized cells using anti-fibronectin (Calbiochem, from Cambridge Bioscience, U.K.) or anti-galactosyl acidic protein (GFAP) (Dako, High Wycombe, U.K.) showed there were few if any fibroblasts present and that at least the majority of the cells were GFAP<sup>+</sup> astrocytes. This method of preparation of brain cells is essentially that of Bruce *et al.* (1984).

**Cytotoxic T cells and cytotoxicity assays.** SFV-specific and alloreactive (anti-H-2<sup>b</sup>) CTL were prepared as previously described (Blackman & Morris, 1985). Brain cell cultures were treated with 1000 (reference research) units/ml of mouse IFN- $\alpha$  (Lee Biomedical, op. act.  $4 \times 10^6$  units/ml, from Strazich Scientific, London, U.K.) for 3 days prior to cytotoxicity assays. Control cultures were left untreated. The cultures were treated with  $^{51}\text{Cr}$  [ $^{51}\text{Cr}$  (DO<sub>2</sub>), from Amersham] at 20  $\mu\text{Ci}/\text{ml}$  overnight and the next morning mock infected or infected with SFV (m.a.i. 30, 300 or 1000 in different experiments). Cells were harvested by treatment with EDTA (0.02%, in phosphate-buffered saline (PBS)) for 15 min at 37°C, counted and distributed into the wells of 96-well microtitre plates at  $2 \times 10^6$  cells/well. SFV-specific effector CTL were harvested and washed and added to the target cells in wells at about 4 h post-infection to give effector:target (E:T) ratios ranging from 1 to 30:1.  $^{51}\text{Cr}$  release was determined after a further 5 h and specific release was calculated by the formula: % lysis =  $100 \times [\text{release in presence of effectors} - \text{spontaneous release}] / [\text{total release} - \text{spontaneous release}]$ , where total release was that in the presence of 0.5 M-hydrochloric acid.

In some experiments cytotoxicity against the lymphoblastoid cell targets EL4, RDM4 (infected or uninfected) or YAC (uninfected) was determined (depending on the numbers of effector cells available) to monitor cytotoxicity observed against the brain cell targets.

Alloreactive CTL killing was determined in essentially the same way but omitting the SFV infection.

**Quantification of surface SFV and MHC class I antigens by specific antibody.** SFV and MHC class I antigen expression on the surface of infected cells was determined in parallel to the cytotoxicity assays. SFV antigens were measured either by  $^{51}\text{Cr}$  release in the presence of SFV-specific antibodies and complement (Pel-Freez, from North-East Biomedical, Didsbury, U.K.), or by indirect immunofluorescence, quantified by flow cytometry. MHC class I antigen expression was also measured by indirect immunofluorescence using antibodies to H-2<sup>b</sup>. The antibodies used were as follows. (i) A hyperimmune rabbit antiserum against SFV prepared by inoculation of purified SFV (Blackman & Morris, 1985). This antiserum showed no reactivity with uninfected brain cells or lymphoblastoid cells but some reactivity with uninfected fibroblasts. (ii) Monoclonal anti-H-2K<sup>b</sup> from hybridoma clone 11.4.1 (ATCC, TIB95). (iii) Monoclonal anti-H-2D<sup>b</sup> from clone 15.5.55 (ATCC, HB24) generously

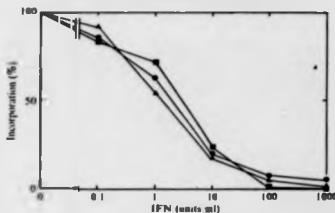


Fig. 1. Effect of IFN- $\alpha$  on incorporation of [ $^3$ H]thymidine into SFV RNA by L929 cells (●), C3H10T1 fibroblasts (▲) and cultured brain cells (■). Incorporation is expressed as a percentage of that by infected cells in the absence of IFN- $\alpha$ , with the background of residual cellular incorporation (i.e. by uninfected cells, 10% or less that of infected cells) subtracted.

provided as peroxidase antibody by E. Culbert (ICI, Macclesfield, U.K.); (v) FITC-conjugated sheep anti-rabbit Ig (Wellcome Diagnostics, Beckenham, U.K.); (vi) FITC-conjugated goat anti-mouse Ig (Mordac Immunological Laboratories, Maidenhead, U.K.). These antibodies were all titrated and used at saturating concentrations. Flow cytometry was carried out using the Becton-Dickinson FACS 440 at Birmingham University, Birmingham, U.K. Cells were harvested and fixed with 3% paraformaldehyde in PBS prior to staining and stored at 4 °C in the dark after staining.

**IFN- $\alpha$  sensitivity of astrocytes.** To determine the relative sensitivity to IFN- $\alpha$  of SFV replication in C3H astrocytes these were distributed in 96-well microtitre plates at  $4 \times 10^5$  cells per well. Two C3H fibroblast lines (L929 and C3H10T1) both of which are highly sensitive to IFN- $\alpha$  were distributed in parallel. Next day the cultures were treated with a laboratory standard IFN- $\alpha$  preparation, 0.1 to 1000 units/ml in tenfold steps. The next day the cells were challenged with SFV (m.o.i. about 300) and labelled from 3 to 5 h post-infection with [ $^3$ H]thymidine (Amersham) in the presence of actinomycin D (Sigma). Under these conditions [ $^3$ H]thymidine is incorporated almost exclusively into SFV progeny RNA when cellular DNA-dependent RNA synthesis is inhibited. TCA-insoluble radioactivity was measured and plotted against IFN- $\alpha$  concentration (Atkins *et al.*, 1974).

## RESULTS

### Relative sensitivity of C3H brain cells to IFN- $\alpha$

Fig. 1 shows the incorporation of [ $^3$ H]thymidine into IFN- $\alpha$  treated, SFV-infected brain cells, L929 cells and C3H10T1 cells. The data show that all three cell types had about equal sensitivity to IFN- $\alpha$ , with about 50% inhibition of virus RNA replication at 1 unit/ml IFN- $\alpha$  and no RNA replication above background levels at 1000 units/ml. Hence 1000 units/ml was used for subsequent experiments.

### Effect of IFN- $\alpha$ treatment on SFV antigen expression in brain cells measured by indirect immunofluorescence

Infection of brain cells with SFV resulted in a marked increase in reactivity with the anti-SFV serum (Fig. 2a and b) with a shift in the mean fluorescence intensity channel (MN) from 30 to 160 and a rise from 4 to 26% in the numbers of cells showing in the maximum fluorescence intensity channel (CH; maximum intensity is channel 255). Pretreatment of the cells with IFN- $\alpha$  however, very markedly reduced SFV antigen display (Fig. 2c) but did not abolish it; a small number of cells were still intensely stained (4% showing in maximum intensity channel) and the mean intensity was clearly elevated over levels seen in the uninfected cells.



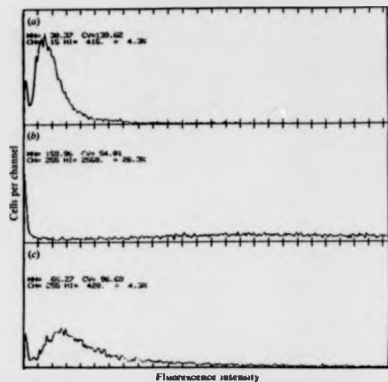


Fig. 2. SFV antigen expression by infected brain cells. SFV antigens were quantified by flow cytometry. Panels (a), (b) and (c) show the fluorescence intensity distribution for untreated, uninfected cells, untreated, infected cells, and IFN- $\alpha\beta$  treated, SFV-infected cells respectively. In each case approximately  $10^5$  cells were processed. MN, mean fluorescence of cells; CV, coefficient of variation; CH, fluorescence channel with largest number of cells in it (i.e. peak fluorescence); N1, number of cells in CH and as a percentage of total cells processed. Fluorescence intensity units are arbitrary, and the scale is linear from channel 0 to 255.

*Effect of IFN- $\alpha\beta$  treatment and SFV infection on expression of MHC class I antigens measured by indirect fluorescence and susceptibility to alloreactive CTL killing*

Analysis by flow cytometry showed that the untreated, uninfected brain cells displayed undetectable levels of H-2K<sup>b</sup> since the fluorescence intensity of anti-H-2K<sup>b</sup>-stained cells was not significantly elevated above that of the same cells stained by an irrelevant antibody of the same class (data not shown). However, as others have found, H-2K<sup>b</sup> levels were markedly increased by IFN- $\alpha\beta$  treatment of these cells (Fig. 3a and b) with a shift in the mean fluorescence intensity channel from 42 to 85. SFV infection itself did not induce H-2K<sup>b</sup> expression in the cultured brain cells (Fig. 3c) nor did SFV infection have any effect on the elevated levels of H-2K<sup>b</sup> expression in IFN- $\alpha\beta$  treated cells (data not shown). Levels of H-2D<sup>b</sup> were similarly determined in brain cells treated with IFN- $\alpha\beta$ . As with H-2K<sup>b</sup>, untreated brain cells showed no detectable H-2D<sup>b</sup> expression since again background fluorescence was essentially the same as for brain cells stained with an irrelevant antibody. However, brain cells treated with IFN- $\alpha\beta$  showed strong expression of H-2D<sup>b</sup>, with mean fluorescence rising from channel 54 to channel 89 (Fig. 4). Hence expression of both major MHC class I antigens is increased in these brain cells by IFN- $\alpha\beta$  treatment.

An indirect measure of H-2 expression by cells is their susceptibility to killing by alloreactive CTL, since these recognize only MHC class I determinants. Accordingly the effect of IFN- $\alpha\beta$

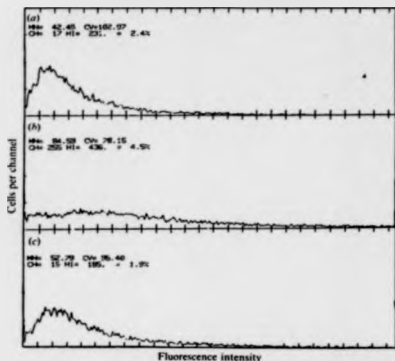


Fig. 3 H-2K antigen expression by IFN- $\alpha\beta$ -treated brain cells. MHC class I antigen (H-2K\*) was quantified by flow cytometry. Panels (a), (b) and (c) show the fluorescence intensity distribution for untreated, IFN- $\alpha\beta$ -treated and SPV-infected cells respectively. In each case approximately  $10^4$  cells were processed. Abbreviations as for Fig. 2.

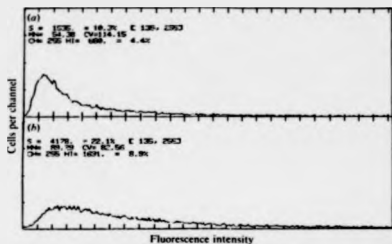


Fig. 4 H-2D expression by IFN- $\alpha\beta$ -treated brain cells. MHC class I antigen (H-2D\*) was quantified by flow cytometry. Panels (a) and (b) show the fluorescence intensity distribution for untreated and IFN- $\alpha\beta$  cells respectively. In each case more than 14000 cells were processed. Abbreviations as for Fig. 2. S, number of cells sampled and as a proportion of the total.

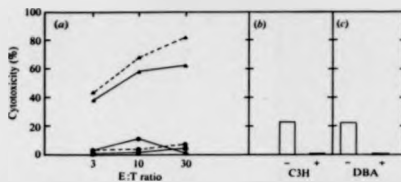


Fig. 5. Effect of IFN- $\alpha$  on lysis of infected brain cells. (a) Lysis by CTL of infected C3H ( $\Delta$ ) or DBA ( $\circ$ ) brain cells, untreated (—) or IFN- $\alpha$ -treated (---), and uninfected non-IFN-treated YAC cells (···) (b and c) Killing of C3H and DBA brain cells respectively by anti-SFV antibody and complement, without (—) and with (+) IFN- $\alpha$ .

Table 1. Lysis of brain cells by alloreactive CTL

Alloreactive CTL line	Cytotoxicity* % specific $^{51}\text{Cr}$ release	
	Without IFN	With IFN- $\alpha$
1 (2nd passage)	4	15
1 (3rd passage)	15	23
2 (3rd passage)	25	63
3 (3rd passage)	1	18

\* Brain cells were treated with IFN- $\alpha$  and subjected to lysis by alloreactive CTL as described in Methods. Data are quoted for an E:T ratio of 10:1.

treatment on susceptibility of brain cells to alloreactive CTL was determined. The data in Table 1 show that in four experiments using three independent alloreactive lines, IFN- $\alpha$  treatment of target cells increased killing, implying an increase in H-2 expression.

*Effect of IFN- $\alpha$  treatment on cytotoxicity of SFV-infected cultured brain cells by SFV-specific CTL or SFV-specific antibody and complement*

In a series of experiments carried out with SFV-specific CTL prepared as above it was regularly found that infected syngeneic brain cells were killed efficiently irrespective of whether they were IFN- $\alpha$ -treated. In most of these experiments uninfected targets were not killed and nor were allogeneic targets (infected or uninfected), hence the killing by these effectors was specific and H-2-restricted as we have previously found with lymphoblastoid targets (Blackman & Morris, 1985). Occasionally the killing was unrestricted, and uninfected cells were also killed, in such experiments YAC cells were also killed, implying the presence of natural killer (NK) cells, and results of these experiments were disregarded. Killing of infected lymphoblastoid targets RDM4 and EL4 was parallel to killing of brain cell targets and quantitatively similar levels of lysis were obtained. In the experiment shown in Fig. 5 killing of infected C3H brain cells with or without IFN- $\alpha$  by CTL and antibody to SFV plus complement is shown. The IFN- $\alpha$  treatment resulted in somewhat increased CTL killing at all E:T ratios but abolished antibody and complement killing. This augmentation of CTL killing by IFN- $\alpha$  treatment of infected targets occurred in eight out of ten experiments; in the other two the killing was undiminished. The experiment shown in Fig. 5 also demonstrated that the CTL killing was MHC-restricted since infected DBA targets (whether or not IFN- $\alpha$ -treated) were not lysed whereas these were lysed by anti-SFV antibody and complement. YAC cells were not lysed, showing that there was no significant NK cell activity in the effector population. Therefore, brain cells treated with IFN- $\alpha$  and infected with SFV were killed by SFV-specific CTL as well

as or better than non-IFN- $\alpha$ -treated brain cells, despite a marked diminution of SFV replication and antigen expression.

#### DISCUSSION

The importance of MHC antigen expression in immune recognition by T cells of their target cells is now well recognized. Cells that express no MHC class I antigens are not lysed by CTL (Zinkernagel & Doherty, 1979). Equally, 'foreign' antigens to which the T cell is sensitized must be present for recognition to occur; hence the T cell recognizes the foreign and self MHC antigen together. Since IFN- $\alpha$  regulates the expression of both viral antigens and MHC antigens, in opposite directions, the effects on CTL lysis of IFN- $\alpha$  treatment of target cells infected with a virus are likely to be complex. In previous experiments with fibroblasts and lymphoblastoid cells we have found that IFN- $\alpha$  treatment usually reduces but does not abolish CTL lysis of SFV-infected cells; others have found in different systems that IFN- $\alpha$  treatment can augment lysis (Bukowski & Walsh, 1985; Flyer *et al.*, 1985). We have extended these experiments in brain cells because these cells normally express very low levels of MHC and so the effects of IFN- $\alpha$  treatment on CTL lysis may be more clear-cut. The study of T cell responses to brain cells is in itself of interest because of the possible role of T cell-mediated immunity in virus encephalitis and encephalopathies such as multiple sclerosis and subacute sclerosing panencephalitis.

We have found that cultured murine brain cells (predominantly GFAP<sup>+</sup> astrocytes) infected with SFV are susceptible to lysis by CTL. The fact that this lysis is MHC-restricted very obviously implies that there is sufficient MHC expression for T cell recognition to occur, although this could not be detected by indirect immunofluorescence. It was possible that infection with SFV induced MHC expression during the cytotoxicity assay thus allowing CTL recognition. Considering the short length of time involved this does seem unlikely; however, retroviruses, at least, can induce MHC antigens (Flyer *et al.*, 1985) and so can coronavirus (Mason *et al.*, 1986) so this also was a formal possibility in the case of SFV infection. The flow cytometric data show that H-2K<sup>b</sup> was not induced by SFV infection, but that does not exclude the possibility that some other restriction element may be involved. However, the simplest explanation for the data is that MHC antigens are present in amounts sufficient for CTL recognition but insufficient for detection by antibody. The experiments in which we have found alloreactive CTL to lyse brain cells tend to confirm the presence of MHC antigens.

The IFN- $\alpha$  treatment of the brain cells reduced SFV replication and SFV antigen expression to a very low level but did not completely abolish SFV antigen expression. H-2 expression on the other hand was increased as was shown by increased staining with monoclonal antibodies reactive with H-2K<sup>b</sup> and H-2D<sup>b</sup> and by increased susceptibility to lysis by alloreactive CTL. The net result was that although lysis by antibody and complement was essentially abolished, CTL lysis was somewhat augmented. This could well play an important role in T cell-mediated mechanisms of SFV-induced encephalitis in mice.

Since our primary brain cell cultures are mixed, although predominantly GFAP<sup>+</sup> astrocytes, we cannot at present determine in which cell type SFV replication mainly occurs nor which type is the major target for CTL. However, since up to 80% of cells displayed SFV antigens and up to 80% lysis by CTL occurred, then at least some of the astrocytes must express virus antigens and are killed. Whether other cells (e.g. oligodendrocytes), making up a minor proportion of the cultures, are also killed remains to be determined.

These results have a number of significant implications for resistance to virus infections, especially for infections of the CNS. Provided IFN is present to increase MHC expression, CTL (or other T cell) responses occur even though virus replication is limited and virus components may be present at very low levels. Once such a response is triggered, it could be self-perpetuating; in response to virus-infected target cells T cells produce IFN- $\gamma$  (Morris *et al.* 1982; Blackman & Morris, 1984) which is particularly effective at augmenting MHC expression and so despite its effect in limiting virus replication the T cell response could continue. Preliminary results employing recombinant murine IFN- $\gamma$  prepared in this laboratory from genetically engineered Chinese hamster ovary cells indicate that IFN- $\gamma$  also augments CTL lysis of IFN-treated, SFV-infected brain cells, in a way very similar to that described above for natural IFN-

a $\beta$  The production of IFN- $\gamma$  by T cells responding to virus-infected brain cells could precipitate autoimmune responses to normal CNS components. Thus, it has been shown that IFN- $\gamma$  augmenting H2-la expression by astrocytes enables these cells to present myelin basic protein (MBP) to T cells (Fierz *et al.*, 1985). T cells thus sensitized to MBP could mount an autoimmune attack on the CNS (Zamvil *et al.*, 1985). Hence, a CNS virus infection could trigger in this way diseases such as multiple sclerosis. In such a case one might expect IFN treatment of multiple sclerosis to exacerbate the disease; however, at least IFN- $\alpha\beta$  does not (Jacobs *et al.*, 1982).

Our results overall emphasize the importance of histocompatibility antigens in immune recognition of virus-infected cells and also suggest an important role for IFN- $\alpha\beta$  and virus infections in autoimmune diseases of the CNS.

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## Role of interferon-gamma in T-cell responses to Semliki Forest virus-infected murine brain cells

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### SUMMARY

Primary brain cell cultures prepared from newborn C3H mice were infected with Semliki Forest virus (SFV) or treated with a  $\beta$ -propiolactone-inactivated preparation of SFV (BPL-SFV). The effects of recombinant interferon-gamma (IFN- $\gamma$ ) treatment on SFV replication, SFV antigen display, major histocompatibility complex (MHC) class I and class II antigen expression, susceptibility to lysis by SFV-specific cytotoxic T lymphocytes (CTL) and the ability to stimulate SFV-specific T lymphocytes to release IFN- $\gamma$  were determined. The IFN- $\gamma$  treatment prevented replication of SFV, as determined by incorporation of [ $^3$ H]uridine into SFV-RNA, and reduced expression of SFV antigens on the cell surface, as determined by lysis with antibody and complement or indirect immunofluorescence. BPL-SFV-treated brain cells expressed no SFV antigen detectable by lysis with antibody and complement or indirect immunofluorescence. IFN- $\gamma$  increased expression of MHC class I and class II antigens, measured by indirect immunofluorescence, susceptibility to killing by alloreactive T-cell lines and ability to stimulate an allogeneic mixed lymphocyte reaction (MLR). Brain cells infected with SFV or treated with BPL-SFV were susceptible to killing by the CTL. The killing was MHC restricted and neither uninfected nor untreated cells were killed. IFN- $\gamma$  treatment prior to SFV infection or BPL-SFV treatment resulted in an augmentation of lysis by the CTL, indicating that even where SFV antigen expression is reduced or present at very low levels, in the context of enhanced MHC class I expression cells remain susceptible to CTL killing. Brain cells treated with BPL-SFV stimulated SFV-specific T cells to release IFN- $\gamma$ . Pretreatment of brain cells with IFN- $\alpha\beta$  or IFN- $\gamma$  prior to BPL-SFV treatment markedly increased the ability of the cells to stimulate the SFV-specific T cells to release IFN- $\gamma$ . Release of IFN- $\gamma$  was MHC restricted and brain cells untreated with BPL-SFV did not stimulate IFN- $\gamma$  release. IFN- $\gamma$  released by T cells stimulated with BPL-SFV-treated brain cells increased class II MHC expression by brain cells as assessed by indirect immunofluorescence.

### INTRODUCTION

There have now been repeated demonstrations that cells of the brain express unusually low levels of antigen encoded by the major histocompatibility complex (MHC) (Viletta & Capra, 1978; Wong *et al.*, 1984; Momburg *et al.*, 1986). This is surprising since class I (H2D, K, L) and class II (IA, IE) MHC antigens play a central role in the immune recognition of foreign antigen by T cells. Thus, with few exceptions, cytotoxic T lymphocytes (CTL) only recognize foreign antigen in association with class I antigen, whereas T-helper (Th) lymphocytes recognize foreign antigen in association with class II antigen (Klein *et al.*, 1981). Several reports have demonstrated that whilst both IFN- $\alpha\beta$  and IFN- $\gamma$  augment class I MHC antigen expression by brain cells, only IFN- $\gamma$  augments class II MHC antigen expression, and then only upon a specific population of brain cells (Wong *et al.*, 1984; Tedeschi, Barrett & Kane, 1986;

Morris *et al.*, 1987). IFN modulation of MHC antigen expression may thereby render these cells competent to initiate and participate in T-cell mediated immune reactions within the brain.

Our interests relate to the ways in which IFNs can influence T-cell responses to brain cells infected with the neurotropic Semliki Forest virus (SFV) via modulation of MHC antigen display. Our previous work has demonstrated that IFN- $\alpha\beta$  treatment of primary brain cells, prior to infection with SFV, results in an augmentation of class I MHC antigen display and in susceptibility to lysis by SFV-specific CTL, despite a marked diminution of SFV replication and antigen display (Morris *et al.*, 1987). We have now extended these observations and found that IFN- $\gamma$  augments CTL lysis of IFN- $\gamma$ -treated SFV-infected brain cells in a similar manner. CTL and Th lymphocytes have been shown to produce IFN- $\gamma$  upon appropriate recognition of a virus-infected cell (Morris, Lin & Askonas, 1982; Cunningham *et al.*, 1985). Therefore, we have studied whether or not our SFV-specific T cells produce IFN- $\gamma$  when stimulated by brain cells treated with a  $\beta$ -propiolactone-inactivated prep-

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ation of SFV (BPL-SFV). It was found that BPL-SFV-treated brain cells stimulated SFV-specific T cells to release IFN- $\gamma$  and that pretreatment of brain cells with either IFN- $\alpha$  or IFN- $\gamma$  prior to treatment with BPL-SFV markedly increased the amount of IFN- $\gamma$  released.

## MATERIALS AND METHODS

### Cells

1929 cells were cultured in the Glasgow modification of Eagles MEM (GEMEM; Gibco, Paisley, Renfrewshire) supplemented with 10% newborn calf serum (Gibco), 2 mM glutamine, penicillin (40 µg/ml) and streptomycin (100 µg/ml). Lymphoblastoid cells EL4 (H2<sup>d</sup>), RDM4 (H2<sup>d</sup>), YAC-1 (H2<sup>k</sup>) (a natural killer cell target) and mastocytoma P15 (H2<sup>d</sup>) were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), heat-inactivated prior to use for 30 min at 56°, with glutamine, penicillin and streptomycin as before. Primary brain cell cultures were prepared from 1-2-day-old C3H/He mice (H2<sup>k</sup>) from breeding pairs supplied by Bausa and Kingman (Aldrough, Hull) as previously described (Morris *et al.*, 1987). Indirect immunofluorescence of paraffin-embedded cells using anti-thrombocytin (Calbiochem, Cambridge Bioscience, Cambridge) or anti-glial-fibrillary acidic protein (GFAP) (Dako, High Wycombe, Bucks) showed there were few if any thrombocytin-containing thrombocytes present and that at least the majority of cells present were GFAP<sup>+</sup> astrocytes.

### Viruses

The avirulent strain of SFV A774 (Bradish, Allner & Maher, 1971) was used for the immunization of mice and the virulent L10 strain for *in vitro* infection of target cells. Stocks of these viruses were prepared as described previously (Blackman & Morris, 1984). For experiments with inactivated virus, the stock L10 virus was treated with  $\beta$ -propiolactone (BPL). Virus was dialysed overnight against 50 mM Tris-HCl (Sigma, Poole, Dorset), pH 7.25 then incubated with 0.1% BPL (Sigma, grade II) for 5 hr at 4°, followed by overnight dialysis against the same Tris buffer to remove residual BPL and its breakdown products, prior to final dialysis back into GEMEM. This procedure reduced the infectivity of SFV from 10<sup>6</sup> plaque-forming units (PFU)/ml to less than 10 PFU/ml and rendered the virus unable to undergo replication, as assessed by incorporation of [<sup>3</sup>H]uridine into viral RNA in infected cells.

### Interferons

Murine IFN- $\alpha$ , specific activity 4 × 10<sup>6</sup> U/ml, was purchased from Lee Biomolecular, San Diego, CA. Murine IFN- $\gamma$  was prepared from Chinese hamster ovary cells (G. D. Searle, High Wycombe, Bucks) transfected with the mammalian expression vector pKSV4 containing a cDNA copy of the Murine-IFN- $\gamma$  mRNA. Details of the procedures used are published elsewhere (Morris & Ward, 1987). The supernatant harvested from the transfected cells contained about 1000 U/ml Murine-IFN- $\gamma$ , which was partially purified by affinity chromatography on cibacron blue Sepharose (Sigma) to a specific activity of about 10<sup>6</sup> U/mg protein.

**Preparation of SFV-specific and alloreactive effector T cells**  
SFV-specific lymphocytes were prepared as described previously (Blackman & Morris, 1985). For alloreactive effectors

BALB/c (H2<sup>d</sup>) mice (Ola, Bicester, Oxon) were primed i.p. with 1 × 10<sup>6</sup> C3H/He splenocytes. Ten days later single-cell suspensions were prepared from the spleens of these primed BALB/c mice and also C3H/He mice. The C3H/He cells (stimulators) were treated with mitomycin C (Sigma) at 25 µg/ml in PBS for 45 min at 37°, followed by four washes in medium, to prevent their proliferation. The C3H/He spleen cells were then mixed with the primed BALB/c cells in RPMI medium (supplemented as before and including 10<sup>-5</sup> M  $\beta$ -mercaptoethanol) with 25 × 10<sup>6</sup> of each cell type in 25 ml. For cytotoxicity and proliferation assays, cells were harvested at 5 and 10 days, respectively, and spun over lymphoprep (Nyegaard, Torskov, Norway) prior to use, in order to remove cell debris.

### Cytotoxicity assays and production of IFN by effector T cells

Two days prior to cytotoxicity assays, brain-cell cultures were treated with 100 U/ml (laboratory units) murine IFN- $\gamma$ . Control cultures were left untreated. Cultures were treated with <sup>51</sup>Cr [<sup>51</sup>Cr<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>; Amersham, Aylesbury, Bucks] at 20 µCi/ml overnight and the next morning mock infected or infected with SFV or BPL-SFV [multiplicity of infection (MOI) 1000]. At 2 hr post-infection, cells were harvested by treatment with EDTA (0.02% in PBS) for 15 min at 37°, then distributed into wells of 96-well round-bottomed microtitre plates at 1 × 10<sup>6</sup> cells/well. SFV-specific effectors were then added to target wells at 4 hr post-infection, to give effector:target ratios (E:T) of 3:1 to 30:1. <sup>51</sup>Cr release was determined 5 hr later and specific release calculated using the formula:

$$\% \text{ lysis} = \frac{100 \times (\text{release in presence effectors} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})}$$

where total release is in the presence of 0.5 M hydrochloric acid. Cytotoxicity against lymphoblastoid cells was determined in order to monitor killing. Previous experiments have shown CTL prepared in this way to be antigen specific and MHC restricted (Morris *et al.*, 1987; Blackman & Morris, 1984, 1985). Alloreactive killing was determined in essentially the same way as for SFV-specific CTL assays but omitting SFV infection.

In order to monitor the production of IFN by SFV effector T cells stimulated by brain cells, the assay was carried out in the same way except that target cells were not labelled with <sup>51</sup>Cr; the E:T ratio was 10:1, cells were only treated with BPL-SFV (since live virus would interfere with the subsequent IFN assay), and some brain cells were treated with 1000 U/ml murine IFN- $\alpha$  (related to NIH mouse IFN standard G002-904-511). The supernatant medium was collected after 6 hr, centrifuged at 10,000 g for 2 min and the IFN assayed as outlined below.

### Alloproliferation assays

For alloproliferation experiments the assay was set up in essentially the same way as for cytotoxicity assays, except that brain cells were treated with mitomycin C (25 µg/ml in PBS) to prevent their proliferation prior to the addition of the alloreactive responders. After 30 hr co-culture [<sup>3</sup>H]thymidine (Amersham) was added to each well to a final concentration of 3 µCi/ml. After a further 24 hr cells were harvested and amounts of [<sup>3</sup>H]thymidine incorporated measured.

### IFN assays and IFN- $\gamma$ sensitivity of brain cells

IFN was assayed by the INAS<sub>2</sub> method (Aitkins *et al.*, 1974)

using SFV challenge of  $L_{10}$  cells. IFN activity was characterized by neutralization assay using a polyclonal rabbit antiserum to IFN induced by Newcastle Disease virus in  $L_{10}$  cells (IFN  $\alpha + \beta$ ), and by the monoclonal antibody anti-Mu-IFN- $\gamma$  (R4-6A2, E. Havell, Trudeau Institute, New York). The relative sensitivity of brain cells to IFN- $\gamma$  was determined as previously described (Morris *et al.*, 1987).

#### Quantification of surface SFV and MHC antigens

SFV antigens were measured in parallel to the cytotoxicity assays either by  $^{51}\text{Cr}$  release in the presence of SFV-specific antibodies and complement (Pel-frez, North-East Biomedical, Uxbridge, Middlesex) or by indirect immunofluorescence, quantified by flow cytometry. MHC class I and II antigen expression were also measured by indirect immunofluorescence. Antibodies used were (i) a hyperimmune rabbit antiserum against SFV prepared by inoculation of purified SFV (Blackman & Morris, 1985); (ii) monoclonal antibody anti-H2-K\* from hybridoma clone 11-6-1 (ATCC, TIB95); (iii) monoclonal anti-H2-D\* from clone 15-5-55 (ATCC, HB24) provided as purified antibody by E. Culbert (ICI, Macclesfield); (iv) monoclonal anti-H2-1A\* from clone 10-3-6 (ATCC, TIB92); (v) monoclonal anti-H2-K\* from clone 11-3-45 (Uniscience, London); (vi) FITC-conjugated sheep anti-rabbit Ig (Wellcome Diagnostic, Beckenham, Kent); (vii) FITC-conjugated goat anti-mouse Ig (Nordie Immunological Laboratories, Maidenhead, Berks). All antibodies were titrated and used in saturating concentrations. After staining, cells were fixed in 3% paraformaldehyde in PBS and stored in the dark at 4°C prior to flow cytometry analysis using the Becton-Dickinson FACScan at Birmingham University. Fluorescence data were collected in list mode using linear amplification and analysed subsequently. Histograms of cell number against fluorescence intensity were printed. The number of fluorescing cells were determined by setting a marker on the histograms at the fluorescence intensity channel for control sample, stained with an irrelevant antibody, at a point where only 3% of the cells processed were of higher fluorescence. For each sample thereafter all cells of higher fluorescence than the marker were deemed above background fluorescence.

## RESULTS

#### Relative sensitivity of C3H brain cells to IFN- $\gamma$ and effect of IFN- $\gamma$ treatment on SFV antigen expression measured by indirect immunofluorescence

In order to assess the relative sensitivity of C3H brain cells to IFN- $\gamma$ , the incorporation of [ $^3\text{H}$ ]uridine into IFN- $\gamma$ -treated SFV-infected brain cells and  $L_{10}$  cells was determined. It was found that both cell types had approximately equal sensitivity to IFN- $\gamma$ , with 50% inhibition of virus RNA synthesis at 1 U/ml IFN- $\gamma$  and no RNA synthesis above background levels at 100 U/ml (data not shown). One-hundred units per millilitre IFN- $\gamma$  were therefore used in subsequent experiments.

Infection of brain cells with live SFV markedly increased reactivity with the anti-SFV serum (Fig. 1a and c), with a shift in mean fluorescence from 6 to 110, peak channel from 3 to 255, and an increase in the percentage of cells above channel 9 from 9% to 95%. In contrast, treatment of the brain cells with an

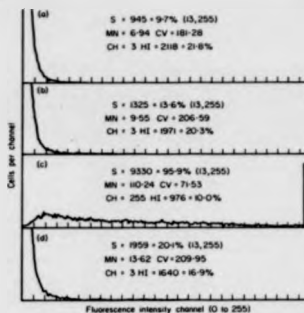


Figure 1. SFV antigen display by infected brain cells. Panels show the fluorescence intensity distribution for (a) uninfected, uninfected cells, (b) BPL-SFV-treated cells, (c) SFV-infected cells, and (d) IFN- $\gamma$ -treated cells. In each case 8728 cells were processed. Abbreviations: S, number of cells above marker and as percentage of total cells processed; CV, coefficient of variation; CH, fluorescence channel with largest number of cells in it (i.e. peak fluorescence); HI, number of cells in CH and as percentage of cells processed. Fluorescence intensity units are arbitrary, and the scale is linear from channel 6 to 355.

equivalent amount of BPL-SFV resulted in essentially no increase in reactivity with anti-SFV serum (Fig. 1a and b). IFN- $\gamma$  pretreatment of brain cells prior to infection with live SFV reduced SFV antigen expression to a level only slightly above background fluorescence (Fig. 1a, c and d).

#### Effect of IFN- $\gamma$ treatment on expression of class I and class II MHC antigens by C3H brain cells, as measured by indirect immunofluorescence

Analysis by flow cytometry showed that untreated brain cells express undetectable levels of H2K\* since the fluorescence intensity distribution of anti-H2K\*-stained cells was not significantly higher than that of the same cells stained with an irrelevant antibody anti-H2K\* (Fig. 2a and b). However, as others have demonstrated (Wong *et al.*, 1984), IFN- $\gamma$  treatment markedly increases H2K\* expression by the cells (Fig. 2b and c), with a shift in mean fluorescence from 11 to 162, peak channel from 3 to 255, and the percentage of cells above the marker increasing from 8% to 93%. Levels of H2-D\* expression were determined in the same manner. At least some untreated brain cells displayed low levels of H2-D\* since the fluorescence intensity of cells stained with anti-H2-D\* was slightly higher than for the same cells stained with the irrelevant antibody (Fig. 3a and b), with a shift in mean fluorescence from 26 to 41, and the percentage of cells above background fluorescence marker



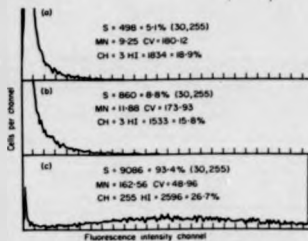


Figure 3. H2-K expression by IFN- $\gamma$ -treated brain cells. MHC class I antigen (H2-K) was quantified by flow cytometry. Panel (a) shows the background fluorescence intensity distribution. Panels (b) and (c) show the fluorescence intensity distribution for untreated and IFN- $\gamma$ -treated brain cells, respectively, stained with anti-H2K. In each case 9728 cells were processed. Abbreviations as for Fig. 1.

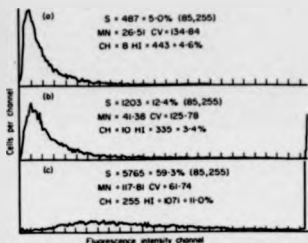


Figure 3. H2-D expression by IFN- $\gamma$ -treated brain cells. MHC class I antigen (H2-D) was quantified by flow cytometry. Panel (a) shows the background fluorescence intensity distribution. Panels (b) and (c) show the fluorescence intensity distribution for untreated and IFN- $\gamma$ -treated brain cells, respectively, stained with anti-H2D. In each case 9728 cells were processed. Abbreviations as for Fig. 1.

rising from 5% to 12%. IFN- $\gamma$  treatment of brain cells considerably increased H2-D $\alpha$  expression (Fig. 3b and c), with a shift in peak channel from 10 to 255, and a rise in the mean fluorescence from 41 to 117. IFN- $\gamma$  treatment did not increase binding of the irrelevant antibody (data not shown). IFN- $\gamma$  treatment therefore increases expression of both major MHC class I antigens. Concentrations of IFN- $\gamma$  as low as 0.1 U/ml were found to result in a distinct enhancement of H2D $\alpha$  and H2K $\alpha$  expression, although enhancement was actually maximum at 10 U/ml (data not shown). SFV or BPL-SFV infection itself did not

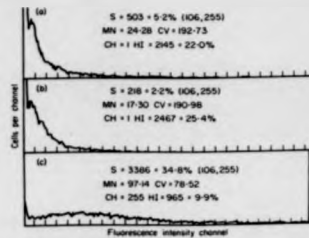


Figure 4. H2-1A $\alpha$  expression by IFN- $\alpha$  and IFN- $\gamma$ -treated brain cells. MHC class I antigen expression was quantified by flow cytometry. Panels show the fluorescence intensity distribution for (a) untreated, (b) IFN- $\gamma$ -treated and (c) IFN- $\alpha$ -treated cells stained with anti-H2-1A $\alpha$ . In each case 9728 cells were processed. Abbreviations as for Fig. 1.

augment class I antigens on the cultured cells, nor have any effect upon the elevated levels of expression on IFN- $\gamma$ -treated cells (data not shown).

Analysis by flow cytometry also showed that the untreated brain cells displayed undetectable levels of H2-1A $\alpha$  since the fluorescence intensity of anti-H2-1A $\alpha$ -stained cells was essentially identical to the same cells stained with an irrelevant antibody (data not shown). Treatment with IFN- $\alpha$  had no effect upon class II MHC antigen expression since the fluorescence intensity distribution of IFN- $\alpha$ -treated cells was essentially identical to that of untreated cells (Fig. 4a and b). However, treatment of brain cells with IFN- $\gamma$  greatly increased the fluorescence intensity distribution with a rise in peak channel from 1 to 255 and a mean shift from 17 to 97 (Fig. 4b and c), showing that IFN- $\gamma$  treatment increases expression of MHC class II antigen by these cultured brain cells. Concentrations of IFN- $\gamma$  at 1 U/ml gave distinct enhancement of H21A $\alpha$  expression and enhancement was maximal at 10 U/ml (data not shown).

#### Effect of IFN- $\alpha$ and IFN- $\gamma$ treatment of brain cells upon class I and class II MHC expression, as assessed by alloreactive lymphocytes

An indirect measure of class I MHC expression by cells is their susceptibility to lysis by alloreactive CTL, since these recognise only MHC class I determinants. Consequently the effect of IFN- $\gamma$  treatment upon the susceptibility of the brain cells to alloreactive CTL lysis was determined. Figure 5 shows that untreated brain cells were slightly more susceptible to alloreactive CTL than control cells of the wrong haplotype (P15), indicating that at least some brain cells express sufficient MHC class I antigen for recognition. IFN- $\gamma$  treatment of the brain cells increased susceptibility, implying increased MHC class I expression. As reported previously (Morris *et al.*, 1987) IFN- $\gamma$  treatment also increased killing, also implying increased MHC

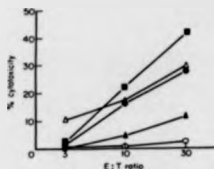


Figure 5. Effect of IFN- $\gamma$  on lysis of brain cells by alloreactive (H2<sup>d</sup> anti-H2<sup>k</sup>) CTL. Untreated (a-a), IFN- $\gamma$ -treated (b-b), IFN- $\gamma$ -treated (c-c), untreated P815 (H2<sup>d</sup>) cells (d-d) and RDM4 (H2<sup>d</sup>) cells (a-a).

Table 1. Ability of brain cells to stimulate an allogeneic MLR

Stimulator brain cell	Responders		Stimulator
	10:1	3:1	Alone
Untreated	8928 $\pm$ 1293	7025 $\pm$ 1211	4093 $\pm$ 1675
IFN- $\gamma$ treated	8815 $\pm$ 1308	8959 $\pm$ 1913	2912 $\pm$ 1373
IFN- $\gamma$ treated	29971 $\pm$ 2074	26993 $\pm$ 2838	2674 $\pm$ 380

Results were the mean of quadruplicate cultures and are expressed as mean  $\pm$  SD of [<sup>3</sup>H]thymidine incorporation. Responders were (H2<sup>d</sup> anti-H2<sup>k</sup>) allozyme

class II expression. All killing was H-2 specific since RDM4 cells (H2<sup>k</sup>) were lysed whereas P815 cells (H2<sup>d</sup>) were not.

A measure of functional MHC class II expression can be obtained by assaying the ability of cells to stimulate an allogeneic MLR. Hence the ability of these brain cell cultures to stimulate an MLR has been investigated. Treatment of brain cells with IFN- $\gamma$  did not increase the ability to stimulate an allogeneic MLR, since levels of [<sup>3</sup>H]thymidine incorporated by responders stimulated by untreated and IFN- $\gamma$ -treated cells were identical (Table 1). In contrast, cells treated with IFN- $\gamma$  clearly stimulated an allogeneic MLR with levels of [<sup>3</sup>H]thymidine incorporated by the responders rising from 8920 to 29971 c.p.m. (Table 1). This increase in functional class II MHC upon treatment with IFN- $\gamma$  but not IFN- $\alpha$  parallels the increases in H-2-IA<sup>b</sup> expression determined by flow cytometry analysis.

#### Effect of IFN- $\gamma$ treatment on lysis of SFV-infected brain cells by SFV-specific CTL or SFV-specific antibody and complement

Figure 6a illustrates that SFV-infected or BPL-SFV-treated brain cells are susceptible to SFV-specific CTL lysis. Untreated SFV-infected brain cells were found to be more susceptible to CTL lysis than untreated, BPL-SFV-treated cells, and in a series of experiments it was found that IFN- $\gamma$  treatment of SFV-infected or BPL-SFV-treated brain cells increased CTL killing. Uninfected syngeneic and infected allogeneic targets were not killed nor were YAC-1 cells, hence all killing was specific, H-2 restricted and not mediated by natural killer cells. As shown in

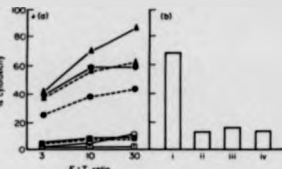


Figure 6. Effect of IFN- $\gamma$  on lysis of infected brain cells. (a) Lysis by CTL of SFV-infected brain cells, untreated (a-a) or IFN- $\gamma$  treated (a-a); BPL-SFV-treated brain cells, untreated (b-b) or IFN- $\gamma$  treated (b-b); uninfected brain cells, untreated (c-c) or IFN- $\gamma$  treated (c-c); SFV- or BPL-SFV-infected ELA cells (c-c) or uninfected, untreated YAC-1 cells (c-c). (b) Killing of brain cells by anti-SFV and complement: (i) SFV infected; (ii) IFN- $\gamma$  treated, SFV infected; (iii) BPL-SFV treated; (iv) untreated, uninfected.

Fig. 6b, treatment of brain cells with IFN- $\gamma$  prior to infection with SFV reduced lysis by SFV-specific antibody and complement to essentially background levels. BPL-SFV-treated brain cells were not susceptible to antibody and complement lysis. Therefore brain cells treated with IFN- $\gamma$  and infected with SFV are more susceptible to lysis by SFV-specific CTL than untreated, SFV-infected cells and this occurs despite a marked reduction in SFV antigen display. Also, BPL-SFV-treated brain cells are susceptible to lysis by SFV-specific CTL, despite no SFV antigen display detectable by antibody.

#### The effect of IFN- $\alpha$ and IFN- $\gamma$ treatment of BPL-SFV-treated brain cells upon the ability to stimulate lymphocytes from SFV-immune mice to produce IFN- $\gamma$

The data in Table 2 show that these brain cells produce small amounts of IFN upon treatment with BPL-SFV. The IFN type produced appears to be IFN- $\alpha$  since activity was neutralized by specific antibodies to IFN- $\alpha$  but not IFN- $\gamma$ . Control brain cells, untreated with BPL-SFV, did not produce any detectable IFN. The ability of these brain cells to stimulate IFN production by SFV-specific lymphocytes was also assessed. As shown in Table 2, supernatants harvested from co-cultures of IFN-untreated, BPL-SFV-treated brain cells with specific lymphocytes contained small amounts of IFN- $\gamma$ , since some activity was neutralized by the monoclonal antibody to IFN- $\gamma$  (which did not neutralize any IFN- $\alpha$  activity in control experiments; data not shown). Small amounts of IFN- $\alpha$  activity were also detected in these supernatants at levels which correspond to the background production of IFN- $\alpha$  due to BPL-SFV treatment of the brain cells. Supernatants harvested from co-cultures containing IFN- $\alpha$ -treated, BPL-SFV-treated brain cells were found to contain significantly (Student's *t*-test,  $P < 0.001$ ) higher titres of IFN activity than those supernatants from co-cultures containing IFN-untreated, BPL-SFV-treated brain cells. Likewise, supernatants from co-cultures of IFN- $\gamma$ -treated, BPL-SFV-treated brain cells possessed even higher levels of IFN activity, which could be neutralized to background levels using antibodies to IFN- $\gamma$ . The titres of IFN detected in supernatants

Table 2. IFN- $\alpha$  and IFN- $\gamma$  potentiation of BPL-SFV-treated brain cells increases the ability to stimulate SFV-specific lymphocytes to release IFN- $\gamma$

Brain cell treatment	Supernatants from co-culture of brain cells with SFV lymphocytes			Supernatants from culture of brain cells only		
	Alone	+ Anti-IFN- $\alpha$	+ Anti-IFN- $\gamma$	Alone	+ Anti-IFN- $\alpha$	+ Anti-IFN- $\gamma$
IFN- $\gamma$ + BPL-SFV	131 $\pm$ 14*	82 $\pm$ 6	28 $\pm$ 9	32 $\pm$ 6	<3	32 $\pm$ 7
BPL-SFV alone	20 $\pm$ 1	16 $\pm$ 1	<3	3	<3	3
IFN- $\alpha$ + BPL-SFV	72 $\pm$ 14	57 $\pm$ 11	<3	10 $\pm$ 7	<3	3
IFN- $\gamma$ alone	<3	ND	ND	<3	ND	ND
IFN- $\alpha$ alone	<3	ND	ND	<3	ND	ND
Untreated	<3	ND	ND	<3	ND	ND

Supernatants were harvested from cultures of brain cells, treated as indicated, in the presence and absence of SFV-specific lymphocytes. IFN activity was assayed and characterized as described in materials and methods.

\* Values refer to mean of six replicate treatments  $\pm$  SD U/ml IFN activity.

ND, not done.

harvested from co-cultures of SFV-specific lymphocytes with either IFN- $\alpha$ -treated or IFN- $\gamma$ -treated brain cells (also treated with BPL-SFV) were significantly different (Student's *t*-test;  $P < 0.001$ ). The production of IFN- $\gamma$  was also MHC restricted since WEHI3b cells (H2<sup>b</sup>) treated in a similar manner did not stimulate the lymphocytes to produce IFN- $\gamma$  (data not shown).

The effect of each of these supernatants upon MHC class II (H2-IA<sup>b</sup>) expression on brain cell cultures was investigated. Table 3 shows that all supernatants harvested from wells containing brain cells only had no effect on class II expression, since the fluorescence intensity of cells treated with these supernatants was essentially identical to background fluorescence. Supernatants harvested from co-cultures of SFV-specific lymphocytes and brain cells untreated with BPL-SFV (irrespective of any treatment with IFN- $\alpha$  or IFN- $\gamma$ ) also had no effect upon MHC class II expression. However, treatment of brain cells with supernatants harvested from co-cultures containing BPL-SFV-treated, IFN-untreated brain cells significantly increased H2-IA<sup>b</sup> expression, with a shift in mean fluorescence from 13 to 40 and its percentage of cells above background marker from 5 to 26. Those supernatants harvested from co-cultures containing BPL-SFV-treated, IFN-treated brain cells elevated the fluorescence intensity to even higher levels, with a rise in the mean from 13 to 53 and the percentage of cells above background marker from 5 to 33. Hence BPL-SFV-treated brain cells stimulate SFV-specific lymphocytes to produce IFN- $\gamma$ , which augments MHC class II expression by brain cell cultures. Preliminary studies have indicated that the supernatants can also augment class I MHC expression by brain cells.

## DISCUSSION

We have shown previously that IFN- $\alpha$  treatment of lymphoblastoid or primary brain cells prior to infection with SFV results in an augmentation of class I MHC antigen display and of lysis by SFV-specific CTL, despite a marked diminution of SFV replication and antigen display (Morris *et al.*, 1987; Blackman & Morris, 1985). We have now extended these studies in order to determine whether or not IFN- $\gamma$  augments CTL lysis of IFN- $\gamma$ -treated, SFV-infected brain cells in a similar manner.

Table 3. Effect of IFN produced by the co-culture of brain cells and SFV-specific lymphocytes upon class II MHC (H2-IA<sup>b</sup>) expression by brain cell cultures

Brain cell treatment	Fluorescence intensity distribution			
	Supernatant from brain cells and lymphocytes		Supernatant from brain cells only	
	MN*	%†	MN	%
IFN- $\gamma$ + BPL-SFV	40	30	10	3
BPL-SFV alone	40	26	14	7
IFN- $\alpha$ + BPL-SFV	53	33	14	6
IFN- $\gamma$ alone	17	9	19	10
IFN- $\alpha$ alone	18	10	14	6
Untreated	18	9	19	10

Supernatants were harvested from wells containing brain cells treated as indicated, and cultured in the presence or absence of SFV-specific lymphocytes. Supernatants were then diluted 1:4 prior to addition to brain cell cultures for 48 hr at 37°. Cells were then harvested and stained by indirect immunofluorescence for class II (H2-IA<sup>b</sup>) MHC antigen expression, quantitated by flow cytometry.

Background fluorescence: MN = 13, % = 5.

\* MN, mean fluorescence.

† Percentage of cells above 5% background fluorescence marker.

Our data show that brain cells (predominantly GFAP<sup>+</sup> astrocytes) infected with SFV are susceptible to SFV-specific CTL lysis. Since killing was MHC restricted, this implies that there was sufficient class I MHC antigen present for T-cell recognition to occur, although this could not be detected by indirect immunofluorescence. Experiments in which alloreactive CTL were found to lyse brain cells also imply the presence of at least low levels of class I MHC antigen. IFN- $\gamma$  treatment of brain cells reduced SFV replication in the cells to a level undetectable via the incorporation of <sup>3</sup>H uridine into SFV-RNA. Likewise IFN- $\gamma$  reduced SFV antigen expression to a

level undetectable by indirect immunofluorescence or by lysis using antibodies to SFV in the presence of complement. In contrast, IFN- $\gamma$  greatly increased expression of class I MHC antigen, as assessed by an increased susceptibility of IFN- $\gamma$ -treated brain cells to lysis by alloreactive CTL. Indirect immunofluorescence studies demonstrated that IFN- $\gamma$  greatly augments expression of both major class I MHC antigens, H2D<sup>b</sup> and H2K<sup>b</sup>. IFN- $\gamma$  treatment of the brain cells prior to SFV infection resulted in an augmentation of lysis by the SFV-specific CTL, indicating that even where SFV antigen expression is greatly reduced, in the context of enhanced MHC class I expression, brain cells remain susceptible to CTL lysis. This may play an important role in T-cell mediated mechanisms of SFV-induced encephalitis in mice. Bearing in mind IFN- $\gamma$  treatment of brain cells essentially abolished SFV replication and antigen display, yet cells remained susceptible to SFV-specific CTL lysis, the question arises as to the source of viral antigen recognized by the CTL. Experiments in which brain cells treated with BPL-SFV were found to be susceptible to lysis by SFV-specific CTL, despite expressing no SFV antigen detectable by indirect immunofluorescence or antibody and complement lysis, suggests that the input viral antigen with which cells are challenged may be recognized by CTL.

IFN- $\gamma$  treatment of the brain cells increased expression of class II (IA<sup>b</sup>) MHC antigen from otherwise undetectable levels, as assessed by indirect immunofluorescence. This increase was found to represent a functional increase in expression which could be recognised by Th cells, since IFN- $\gamma$ -treated brain cells were found to stimulate an allogeneic MLR. Treatment of the brain cells with IFN- $\gamma$  had no effect upon class II expression as assessed by indirect immunofluorescence or ability to stimulate an allogeneic MLR.

Our data also show that brain cells treated with BPL-SFV stimulate SFV-specific T cells to release IFN- $\gamma$  and that pretreatment of brain cells with IFN- $\gamma$  prior to BPL-SFV treatment increases the ability of the cells to stimulate SFV-specific T cells to release IFN- $\gamma$ . Similarly, brain cells pretreated with IFN- $\gamma$  were found to stimulate SFV-specific T cells to release even higher titres of IFN- $\gamma$ . A possible explanation for this is that IFN- $\gamma$  and IFN- $\alpha$  augment class I MHC antigen expression and hence the ability of CTL, which release IFN- $\gamma$ , to recognize BPL-SFV-treated brain cells. Furthermore, IFN- $\gamma$ -pretreated BPL-SFV-treated brain cells may stimulate SFV-specific T cells to release higher titres of IFN- $\gamma$  than IFN- $\alpha$ -pretreated BPL-SFV-treated brain cells because IFN- $\gamma$ , unlike IFN- $\alpha$ , augments class II MHC expression on brain cells and thereby increases their ability to be recognized by Th cells, which also release IFN- $\gamma$ . We have also found that IFN- $\gamma$  released by SFV-specific T cells stimulated with BPL-SFV-treated brain cells augments class II MHC (IA<sup>b</sup>) antigen expression on brain cells, as assessed by indirect immunofluorescence. Our brain cell cultures also produced IFN- $\alpha$  when treated with BPL-SFV. The ability of inactivated virus to induce IFN production has been reported previously in other systems (Meager & Burke, 1972).

Overall, our results have a number of significant implications for resistance to virus infections, especially for infections of the CNS. Provided IFN- $\gamma$  is present to increase MHC expression, CTL responses still occur, even though virus replication is limited and virus components may be present at very low levels. The production of IFN- $\gamma$  by T cells responding

to virus-infected brain cells could precipitate autoimmune responses to normal CNS components. Thus it has been shown that IFN- $\gamma$  augmentation of class I MHC antigen expression by astrocytes enables these cells to present myelin basic protein (MBP) to T cells (Fontana, Fiori & Wekerle, 1984; Fiori *et al.*, 1985). T cells thus sensitized to MBP could then mount an autoimmune attack on the CNS (Zanvil *et al.*, 1985). A recent study in which IFN- $\gamma$  treatment of multiple sclerosis patients resulted in an exacerbation of disease (Paratch *et al.*, 1987) emphasizes the necessity for further studies in this direction.

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INTERFERON MODULATION OF T-CELL RESPONSES TO  
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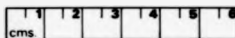
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